Updated: July 23, 2002

Fluorescence Polarization (FP)

Principles

Fluorescence polarization measurements provide information on molecular orientation and mobility and processes that modulate them, including receptor–ligand interactions, proteolysis, protein–DNA interactions, membrane fluidity and muscle contraction (**Figure 1**).

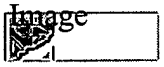


Figure 1. Physical basis of fluorescence polarization assays. Dye molecules with their absorption transition vectors (arrows) aligned parallel to the electric vector of linearly polarized light (along the vertical page axis) are selectively excited. For dyes attached to small, rapidly rotating molecules, the initially photoselected orientational distribution becomes randomized prior to emission, resulting in low fluorescence polarization. Conversely, binding of the low molecular weight tracer to a large, slowly rotating molecule results in high fluorescence polarization. Fluorescence polarization therefore provides a direct readout of the extent of tracer binding to proteins, nucleic acids and other biopolymers.

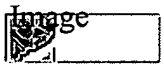
Because polarization is a general property of fluorescent molecules (with certain exceptions such as lanthanide chelates), polarization-based readouts are somewhat less dye-dependent and less susceptible to environmental interferences such as pH changes than assays based on fluorescence intensity measurements. Experimentally, the degree of polarization is determined from measurements of fluorescence intensities parallel and perpendicular with respect to the plane of linearly polarized excitation light, and is expressed in terms of fluorescence polarization (P) or anisotropy (r):



Note that both P and r are ratio quantities with no nominal dependence on dye concentration. Because of the ratio formulation, fluorescence intensity variations due to the presence of colored sample additives tend to cancel and produce relatively minor inteferences. P has physically possible values ranging from −0.33 to 0.5. In practice, these limiting values are rarely attained. Measured values of P in bioanalytical applications typically range from 0.01 to 0.3 or 10 to 300 mP (mP = P/1000). This measurement range is not as narrow as it might appear to be because very precise measurements (P ± 0.002 or ± 2 mP) are readily obtainable with modern instrumentation.

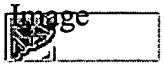
Dependence of Fluorescence Polarization on Molecular Mobility

Interpretation of the dependence of fluorescence polarization on molecular mobility is usually based on a model derived in 1926 from the physical theory of Brownian motion by Perrin.



where P_0 is the fundamental polarization of the dye (for fluorescein, rhodamine and BODIPY dyes, P_0 is close to the theoretical maximum of 0.5), τ is the excited state lifetime of the dye and ρ is the rotational correlation time of the dye or dye conjugate. These relationships can be expressed in terms of fluorescence anisotropy in an equivalent and mathematically simpler manner. For a

hydrodynamic sphere, ρ can be estimated as follows:



where  = solvent viscosity, T = temperature, R = gas constant and V = molecular volume of the fluorescent dye or dye conjugate. In turn, V can be estimated from the molecular weight of the dye or dye conjugate with appropriate adjustments for hydration. Simulations of these relationships are shown in **Figure 2**, leading to the following general conclusions:

- Fluorescence polarization increases as molecular weight increases.
- Fluorescence polarization increases as solvent viscosity increases.
- Fluorescence polarization decreases as the excited state lifetime of the dye (τ) increases.



Figure 2. Simulation of the relationship between molecular weight (MW) and fluorescence polarization (P). Simulations are shown for dyes with various fluorescence lifetimes (τ): 1 ns (cyanine dyes) in purple, 4 ns (fluorescein and Alexa Fluor 488 dyes) in red, 6 ns (some BODIPY dyes) in green and 20 ns (dansyl dyes) in blue. At MW = 1000, P = 0.167 for τ = 1 ns, P = 0.056 for τ = 4 ns, P = 0.039 for τ = 6 ns and P = 0.012 for τ = 20 ns. Simulations assume P_0 (the fundamental polarization) = 0.5 and rigid attachment of dyes to spherical carriers.

Note that these simulations assume that the dye is rigidly attached to a spherical carrier. When conventional parameter estimates for proteins in aqueous solutions are used, ρ is found to increase by about 1 ns per 2400 dalton increase of molecular weight.

Dyes for Fluorescence Polarization Assays

Tracers used in fluorescence polarization assays include peptides, drugs and cytokines that are modified by the attachment of a fluorescent dye. Depolarization due to flexibility in the attachment of the dye, sometimes referred to as the "propeller effect", distorts the relationships between P and molecular weight shown in **Figure 2**. For this reason, it is generally preferable to use reactive dyes without aliphatic linkers between the fluorophore and the reactive group in the preparation of tracers for fluorescence polarization–based assays. A key factor in the performance of fluorescence polarization–based assays is the extent to which the biological activity of the tracer is perturbed by the dye modification. Molecular Probes' BODIPY dyes generally produce less perturbation of receptor-binding affinity and other activity parameters than conventional dyes such as fluorescein and rhodamine. Furthermore, these BODIPY dyes usually have longer excited-state lifetimes than fluorescein and rhodamine dyes, making their fluorescence polarization sensitive to binding interactions over a larger molecular weight range (**Figure 2**). The long-wavelength BODIPY TMR and BODIPY TR dyes also tend to minimize assay interferences due to intrinsically fluorescent sample additives.

Applications

Fluorescence polarization measurements have long been a valuable biophysical research tool for investigating processes such as membrane lipid mobility, myosin reorientation and protein–protein interactions at the molecular level. Immunoassays that have been developed and used extensively for clinical diagnostics represent the largest group of bioanalytical applications. The more recent advent of microplate readers equipped with polarizing optics has led to the adoption of fluorescence polarization as a readout mode for high-throughput screening. Some typical bioanalytical applications of fluorescence polarization–based assays are summarized below.

Examples of flu rescence p larizati n–based assays

Assay Target	Tracer	References
Ligand binding to neurokinin 1 (NK1) receptor	Fluorescein-labeled substance P	Biochemistry 33, 13079 (1994)
Ligand binding to melanocortin G-protein–coupled receptors	BODIPY TMR dye–labeled NDP-αMSH	J Biomol Screen 5, 329 (2000)
Ligand binding to estrogen receptors	Fluorescein-labeled estradiol	J Biomol Screen 5, 77 (2000)
Protein kinase activity	Fluorescently labeled phosphopeptide	Anal Biochem 278, 206 (2000); Methods 22, 61 (2000)
Ligand binding to tyrosine kinase Src homology domains	Fluorescein- and BODIPY TR dye–labeled phosphopeptides	Anal Biochem 275, 62 (1999); Anal Biochem 247, 77 (1997)
Substrate binding to protein farnesyltransferase	Oregon Green 488 dye–labeled peptide	Biochemistry 38, 13138 (1999)
β-lactam antibiotic binding to penicillin-binding proteins	BODIPY FL dye–labeled penicillin V	Antimicrob Agents Chemother 43, 1124 (1999)
Nonspecific protease activity	BODIPY FL dye–labeled casein	Anal Biochem 243, 1 (1996)
Detection of specific PCR products	Fluorescein-labeled oligonucleotide	Gene 259, 123 (2000)
Ligation and cleavage of RNA by ribozymes	Fluorescein- or tetramethylrhodamine-labeled oligoribonucleotide	Biotechniques 29, 344 (2000)
SNP detection by allele-specific primer extension	Fluorescent ddNTP	Genome Res 9, 492 (1999)

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Beacon® 2000 Fluorescence Polarization Systems

Product No.	Description	Price
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P2302**	Beacon® 2000 Fluorescence Polarization System, 220 V / 240 V* (See description for P2300)	**
P2370	Beacon® 2000 Full-range Fluorescence Polarization System, 110 V Full-range Beacon® 2000 FP Instrument (254-700 nm range) with fluorescein filters installed plus complete set of starter reagents (3 Kits) and choice of Option A or Option B.	$36,025
P2372**	Beacon® 2000 Full-range Fluorescence Polarization System, 220 / 240 V* (See description for P2370)	**
P2810	Beacon® 2000 Red Range Fluorescence Polarization System, 110V	$37,025
P2811**	Beacon® 2000 Red Range Fluorescence Polarization System, 220V	**

Option A: Low Volume Chamber (100 µL minimum volume) and Low Volume Accessory Kit*** with 6 x 50 mm tubes

Option B: Standard Volume Chamber (500 µL minimum volume) and Standard Volume Accessory Kit*** with 10 x 75 mm tubes

* Contact PanVera for details on CE Mark.
** Contact your local PanVera distributor for pricing
*** Accessory Kits include the following: 250 Beacon® Disposable Test Tubes (either 6 x 50 mm or 10 x 75 mm); Beacon® 2000 Data Manager Software, ver. 2.2; Thermal Printer (parallel); 2 rolls of Printer Paper; Printer Cable (3' parallel); Computer Cable (6'); Replacement Lamp; Literature Packet (including Instruction Manual).

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The Beacon® 2000 System is an ideal tool for basic research and assay development. Two versions of the System are available. Both provide variable temperature control of the sample from 6°C to 65°C, and a choice of sample chambers requiring a minimum of 100 µL or 500 µL volumes. They are both equipped with external filter holders, thus accommodating a wide range of different wavelength interference filters. The filter pair for use with fluorescein is provided (488 and 535 nm); please contact PanVera for customization of filters for other fluorophores.

The Standard Beacon® 2000

The Standard Beacon® 2000 System uses a quartz-halogen lamp that yields a usable wavelength range of 360-700 nm. All of the assays described in this Chapter may be performed with the Standard Beacon® 2000. For additional capabilities in the ultraviolet range (to 254 nm), and the extended red range (to 900 nm), the Full-Range Beacon® 2000 is available.

The Full-Range Beacon® 2000

The Full-Range Beacon® 2000 System provides ultimate flexibility, with detection capabilities in the ultraviolet and visible ranges (254-700 nm). The instrument may also be ordered with an optional photomultiplier tube (PMT) that extends the emission detection range to 900 nm. The Full-Range System is equipped with both a quartz-halogen and a mercury lamp, and with Glan-Taylor prism polarizers. This extended-range system was designed for applications and fluorophores in the ultraviolet. The Full-Range System fully accommodates all applications of the Standard Beacon® 2000 System. This expanded instrument is required for fluorescence polarization studies in the ultraviolet range, in which fluorophores such as tryptophan, pyrene, dansyl chloride, and IAEDNS are used.



The Beacon® 2000 Fluorescence Polarization Instrument

Beacon® 2000 Instrument Specifications

Beacon® 2000 System Components

Beacon® 2000 System faqs

Fluorescence Polarization PowerPoint® Presentation (.zip - 5.4 MB)
Note: requires archive utility software - download StuffIt Expander™

Fluorescence Polarization Applications Guide



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About Fluorescence Polarization

Fluorescence polarization was first described in 1926 (Perrin) and has been a powerful tool in the study of molecular interactions. When fluorescent molecules are excited with plane polarized light, they emit light in the same polarized plane, provided that the molecule remains stationary throughout the excited state (4 nanoseconds in the case of fluorescein). However, if the excited molecule rotates or tumbles out of the plane of polarized light during the excited state, then light is emitted in a different plane from that of the initial excitation. If vertically polarized light is used to excite the fluorophore, the emission light intensity can be monitored in both the original vertical plane and also the horizontal plane. The degree to which the emission intensity moves from the vertical to horizontal plane is related to the mobility of the fluorescently labeled molecule. If fluorescently labeled molecules are very large, they move very little during the excited state interval, and the emitted light remains highly polarized with respect to the excitation plane. If fluorescently labeled molecules are small, they rotate or tumble faster, and the resulting emitted light is depolarized relative to the excitation plane.

Applications

One of the most widely used fluorescence polarization applications is the competitive immunoassay used for the detection of therapeutic and illicit drugs. A small, fluorescently-labeled drug, when excited with plane polarized light, emits light that is depolarized because the fluorophore is not constrained from rotating during the excitation state. When the labeled drug is added to a serum-antibody mixture, it competes with the unlabeled drug in the sample for binding to the antibody. The lower the concentration of unlabeled drug in the sample, the greater amount of labeled drug that will bind to the antibody. Once bound to antibody, the labeled drug rotates and tumbles more slowly. Light emitted by the fluorescently labeled drug/antibody complex will be more polarized, and the fluorescence polarization value of the sample will be higher. By constructing a standard curve of serum samples with known drug concentrations versus polarization value, the concentration of drug in a patient sample can be easily determined.

Fluorescence Polarization Instruments

The Beacon® 2000 System

The Beacon 2000 FP System is an ideal tool for basic research and assay development. It provides a rapid and precise way to measure equilibrium binding or molecular degradation for a wide range of biological molecules.

Applications for the Beacon 2000 System

- DNA-Protein Binding
- DNA Hybridization and Detection
- Antibody-Antigen Binding
- Protein-Ligand Binding

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Features of the Beacon 2000 System

- Measurement of equilibrium binding directly in solution
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- Variable temperature control of the sample (6 - 65°C)
- External filter holders for use of multiple fluorophores
- Computer-controlled data collection
- Programmable
- Easy to install and use
- Availability of both Standard (360 - 700 nm) and Full-range (254 - 900 nm) Systems

Multiwell Fluorescence Polarization Readers

- TECAN Polarion

Fluorescence Polarization Applications Guide

PanVera Corporation offers a FREE, comprehensive 100+ page FP Applications Guide. This handy resource provides examples of several applications with extensive reference lists for each. It also contains information on labeling methods, analysis of binding data, and experimental considerations and protocols. You can view or download a PDF version of this document online. We would also be happy to send you a printed copy.

2 · 2000

Analytix

Advances in Analytical Chemistry

New Fluorescent Labels for Polarization Assays and Lifetime Imaging



Fluka is pleased to introduce a series of reactive dyes for use in fluorescence polarization assays and fluorescence lifetime imaging applications. Designed specifically to have long fluorescence lifetimes, these dyes were first synthesized in the labs of Dr. Joseph Lakowicz at the Center for Fluorescence Microscopy, University of Maryland Medical School. These laser-excitable metal ligand complexes, based on ruthenium, have fluorescence lifetimes between 400 and 800 nanoseconds [1].

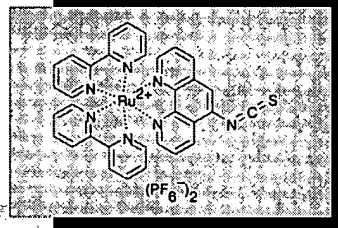
Traditional fluorophores such as fluorescein and rhodamine have fluorescence lifetimes of less than 10 nanoseconds. Dyes with very short lifetimes can be used in fluorescence polarization assays if the molecular weight of labeled antigen is relatively low (M_r < 1,000). On binding to an antibody, the change in the effective molecular weight of the complex is great enough to be reflected as a change in anisotropy. If the molecular weight of the antigen is large (M_r > 20,000), changes in rotational correlation time on Ag:Ab complex formation are small. In this case, fluorophores with short (<10 ns) lifetimes are no longer useful. Dyes with lifetimes in the range of 400 to 500 ns range are better matched to the rotational correlation times of very high molecular weight complexes.

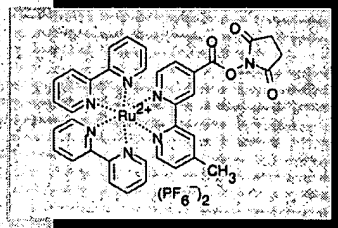
These dyes can be used to label amines on biomolecules under mild conditions. Human serum albumin (HSA) has been labeled with $Ru(bpy)_2dcbpy$, succinimidyl ester (figure 1) by adding a 100-fold excess of the reactive dye in N,N-dimethylformamide (DMF) to a solution of HSA in 0.2 mM carbonate buffer, pH between 8.0 and 9.0. The labeled protein is purified on Sephadex G-25 or G-50 eluting with 0.1 M PBS at pH 7.2. These are sufficient conditions for the labels mentioned below. The resulting conjugate had an emission greater than 540 nm when excited at 483 nm and had a fluorescence lifetime of 336 ns at pH 7.0 [2]. This labeled HSA was used in a model fluorescence polarization immunoassay for polyclonal anti-HSA [3].

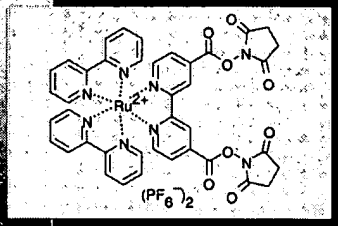
Fluka 71603	Bis-(bipyridine)-5-(isothiocyanato-phenanthrolin)-$Ru(PF_6)_2$
Fluka 96631	Bis-(bipyridine)-4'-methyl-4-carboxy-bipyridine-Ru-N-succinimidyl ester $(PF_6)_2$
Fluka 96632	Bis-(bipyridine)-4,4'-dicarboxy-bipyridine-Ru-di-N-succinimidyl ester $(PF_6)_2$

Advantages of these dyes as protein labels include high photostability, good water solu-

Fig. 1:
A selection of reactive fluorescent ruthenium complexes

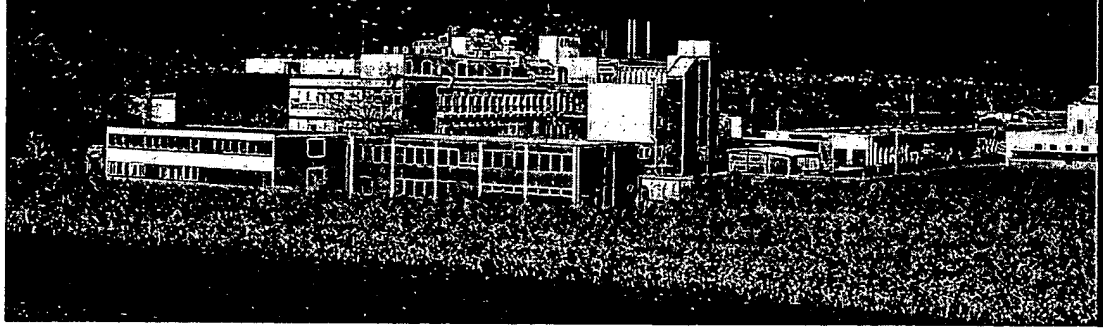






Contents:

- **New fluorescent labels for polarization assays and lifetime imaging**
- **powerful cell-permeable fluorophores for life cell assays**
- **improved ion probes**



Fluka

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bility, a lack of dye-dye interactions and large Stokes' shifts.
In addition, the fluorescence signal of long-lived fluorophores can be gated to eliminate the emission from short-lifetime fluorophores and autofluorescence from cells and biomolecules to further improve sensitivity.

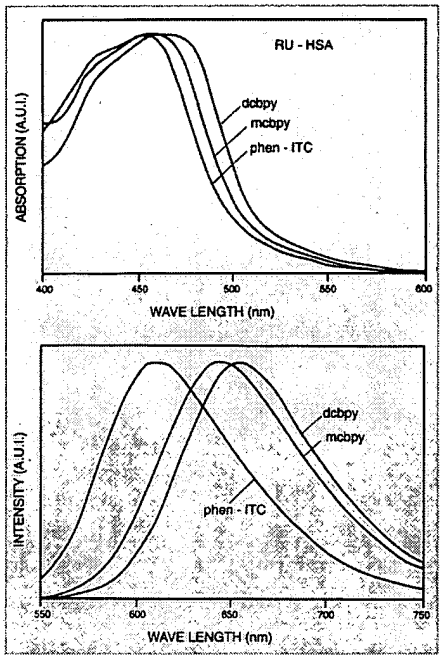


Fig. 2
Absorption and emission spectra of fluorescent ruthenium complexes bound to human serum albumin, 20°C, 10 mM MOPS pH 7.3.
Excitation wave length 460 nm (from [1])

Cell-permeable Fluorophores for Life Cell Assays

We would like to highlight three important fluorescent indicators that have proven to be powerful tools for the direct, real-time imaging of biological events – Calcein-AM, BCECF-AM and Fluo-3 AM. These three dyes form the basis for a wide variety of reported live-cell assays. They are available in a nonpolar form that passively crosses the plasma membrane and accumulates in the cytoplasm of living cells. After cleavage of the protecting groups by intracellular esterases, the polar fluorophore (4–6 negative charges/molecule) remains trapped within the cell. The indicators can be excited at 488 nm. Since the dye is nonfluorescent prior to cleavage, only the dye that has accumulated in living cells gives a fluorescent signal.

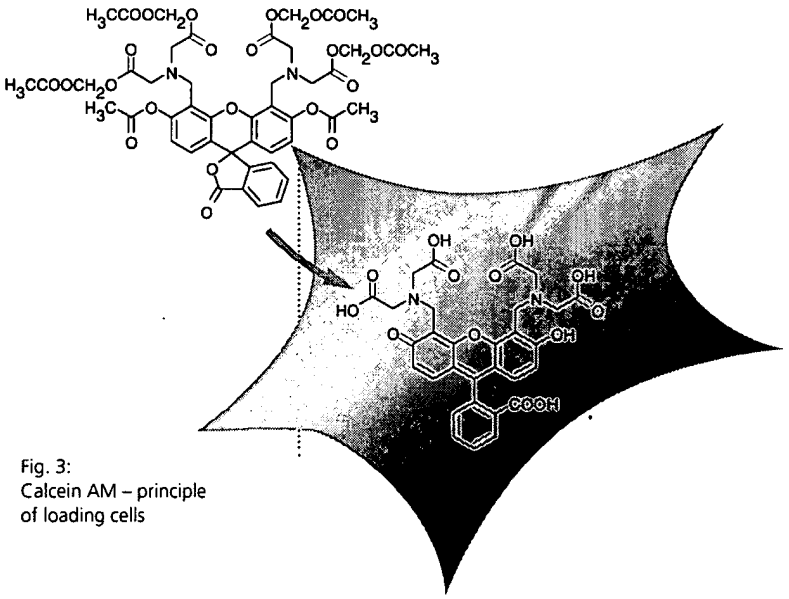


Fig. 3:
Calcein AM – principle of loading cells

Thes indicat rs:

- are excited by the 488 nm argon laser line and used with standard fluorescein filters
- stain only live cells with intact membranes
- accumulate in the cytoplasm to give a large fluorescence signal
- are nonfluorescent in the cell-permeable form, so that there is little or no background fluorescence from the extracellular dye
- can be used with orange and red fluorescent fluorophores for multi-color imaging applications
- can be used to perform cell viability assays using a variety of instruments such as fluorescence well-plate readers, flow cytometers, microscopes and fluorometers.

Loading live cells with AM esters:
Use of these dyes in cell viability assays is straightforward:

1. The protected form of the dye (AM) is dissolved in dry DMSO to give a concentration of 1 millimolar.

2. An aliquot is then diluted into buffer and added to cultured cells to give a final dye concentration of 1 to 50 μM. The cells are bathed in this solution for 30 minutes to 2 hours.

3. The extracellular dye can then be washed away, or if a small background signal is tolerable, the cells can be imaged directly.

Calcein-AM ester has proven to be one of the most important and versatile fluorescent dyes for cell viability research. The dye passively crosses the plasma membrane of living cell where it is cleaved by intracellular esterases to reveal a very polar derivative of fluorescein (calcein) that remains trapped in the cytoplasm.
Calcein can be excited by the argon laser line at 488 nm and, unlike most fluorescein derivatives, its emission intensity is not sensitive to changes in pH over the physiological range.
Assays using Calcein-AM include determining cell viability [4,5] proliferation [6] and adhesion [7]. In NIH 3T3 cells, Calcein-AM has been shown to be more sensitive than Annexin V for detection of the early phases of programmed cell death (apoptosis) [8]. Cytotoxity assays using Calcein AM have been developed as an alternative to radioactive ^{51}Cr-release assays [9].
Cobalt chloride quenches cytoplasmic calcein, and this has been used to monitor the permeability of mitochondrial pores. Fluo-3 AM is a laser-excitable (488 nm) fluorescent calcium indicator. Like Calcein-AM, Fluo-3 AM is nonfluorescent and is converted to the polar Fluo-3, which accu-

mulates in the cytosol. Intracellular Fluo-3 is sensitive to changes in intracellular free calcium [10]. Because of the importance of Ca^{2+} as a messenger, Fluo-3 AM has found widespread utility in high throughput screening assays.
Some assays utilizing Fluo-3 AM in live cells include flow cytometric assays for multi-drug resistance [11,12] and for detecting the expression of Na^{+}/Ca^{2+}-exchanger clones [13].

BCECF-AM is a pH indicator (pKa = 6.98) with an excitation wave length of 488 nm that is useful for imaging changes in cytosolic pH. Like the two dyes above, BCECF-AM itself is nonfluorescent and passively crosses the plasma membrane of cells. BCECF has a pH-insensitive isobestic point at about 527 nm, allowing for ratio imaging of pH changes to correct for differences in path length, dye concentration and bleaching.

Assays have been described that use BCECF-AM in drug screening, cytotoxicity/proliferation [14] and cell viability [15]. The pH response has been used to indirectly determine intracellular potassium levels and monitor nitric oxide (NO)-induced apoptosis. High throughput assays using BCECF include screens for cancer cell attachment [16] and for drugs that alter proton flow through the cell membrane [17].

Related products offered by Fluka:
Ethidium homodimer, unlike Calcein-AM, stains only dead cells. This dye enters cells that have compromised (leaky) membranes and is essentially nonfluorescent until binding to DNA, which elicits a bright red fluorescence. This dye has a much higher affinity for DNA than ethidium bromide. The dye:DNA complexes are extremely stable. In a sensitive cell viability assay, it has been used to detect tissue necrosis factor (TNF) activity[18]. Because ethidium homodimer binds only double-stranded DNA, it has proven useful as an indicator of nucleotide hybridization[19].
Dihydroethidium is a cell-permeable derivative of the DNA stain ethidium bromide. In parts of the cell where there is oxidative activity or stress, dihydroethidium is oxidized to ethidium bromide. The ethidium bromide then binds to intracellular DNA producing a bright red fluorescence. Dihydroethidium has been shown to exhibit increased fluorescence in six models of apoptosis[20]. It has also been used to detect superoxide generation in the mitochondria of living cells[21].

Fluka 17783	Calcein-AM
Fluka 46394	Fluo 3-AM
Fluka 14562	BCECF-AM

Fluka 46043	Ethidium homodimer
Fluka 37291	Dihydroethidium
Fluka11696	BAPTA-AM
Fluka 16569	4-Br-A23187
Fluka 21879	5(6)-Carboxyfluorescein diacetate

BAPTA-AM ester and Br-A23187 are used to calibrate the signal from Fluo-3 in cells. BAPTA-AM chelates intracellular Ca^{2+} resulting in a minimum signal of Fluo-3, whereas Br-A23187 is an ionophore that allows extracellular Ca^{2+} to enter the cytoplasm of live cells, resulting in a maximum Fluo-3 signal.
Like Calcein-AM, the diacetates of 5(6)-carboxyfluorescein can be used as indicators of cell viability. The diacetates are nonfluorescent and are converted intracellularly to the fluorescent carboxyfluorescein on cleavage by esterases. Although these dyes are more sensitive to changes in intracellular pH and are not retained in cells as well as Calcein-AM, they provide a less expensive alternative for the analysis of cell viability over short periods.

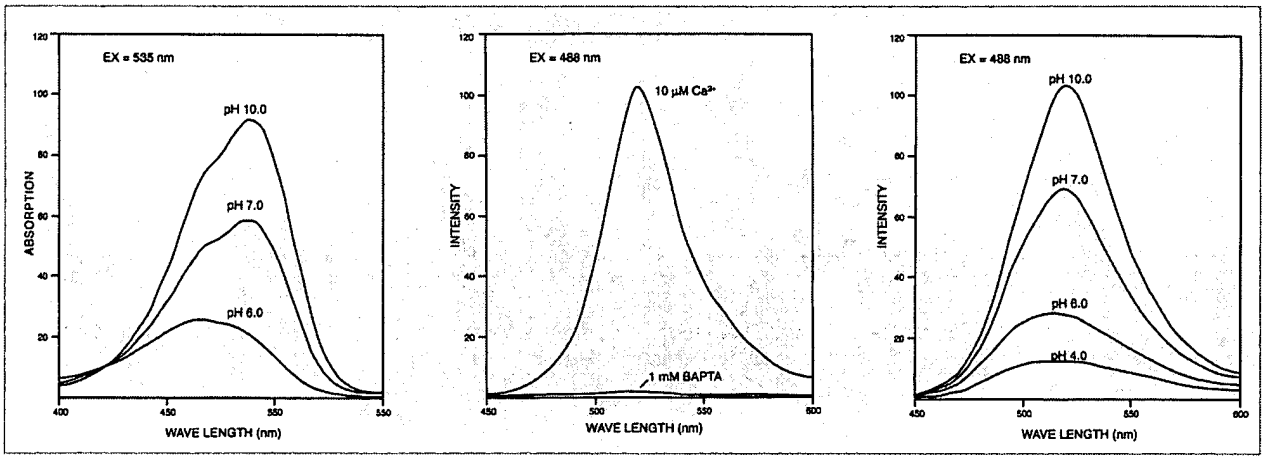


Fig. 4:
BCECF excitation
spectra – response to pH

Fig. 5:
Fluo 3 emission
spectra – response to calcium

Fig. 6:
BCECF emission
spectra – response to pH

Superior Calcium Indicators

Fluorescent indicators have enabled the investigation of changes in intracellular free Ca^{2+} concentrations. Although they are undisputedly the most sensitive tools for this purpose, some problems are associated with the most popular Ca^{2+} indicators, Fura 2, Fluo 3 and Indo 1, in some applications.

High calcium indicators

Name and Product No.	Solubility	Excitation/Emission (bound to Ca^{2+})	Kd
Fluo-3FF* 17022	H_2O	500, 515 / 526	41 µM
Fluo-3FF AM 17079	DMSO	500, 515 / 526 on hydrolysis	
Indo-1FF* 17091	H_2O	350 / 435	33 µM
Indo-1FF AM 17088	DMSO	350 / 435 on hydrolysis	
Fura-2FF* 17085	H_2O	340 / 505	35 µM
Fura-2FF AM 17090	DMSO	340 / 505 on hydrolysis	

*potassium salt

Near membrane calcium indicators

Name and Product No.	Excitation	Emission maximum	Kd
FFP 18* 17089	Free: 335 Bound: 335	Free: 502 Bound: 492	400 nM
FFP 18 AM 17083	As above on hydrolysis	As above on hydrolysis	
FIP 18* 17087	Free: 346 Bound: 346	Free: 475 Bound: 408	450 nM

*potassium salt

Leakage resistant calcium indicators

Name and Product No.	Excitation	Emission maximum	Kd
Fura PE3* 17082	Free: 364 Bound: 335	Free: 502 Bound: 495	250 nM
Fura PE3-AM 17081	As above on hydrolysis	As above on hydrolysis	
Indo PE3* 17084	Free: 350 Bound to Ca^{2+}: 350	Free: 475 Bound: 408	260 nM
Indo PE3-AM 17086	As above on hydrolysis	As above on hydrolysis	

*potassium salt

Furthermore, the use of these indicators is limited, with respect to the range of Ca^{2+}-concentration as well as to the location of Ca^{2+}.
Fluka now offers new calcium indicators that overcome these traditional problems or limitations.

High calcium indicators

Indicators specially modified for measuring high calcium have:

- reduced sequestration of intracellular calcium
- reduced perturbation of calcium transients
- measurement of short-lived transients
- absence of Mg^{2+}-effects

Excitation and emission wave lengths, photostability and quantum yields are comparable to the related low Ca^{2+}-indicators Fura-2, Indo-1 and Fluo-3.

Near membrane calcium indicators

Near membrane indicators associate primarily with the plasma membrane. FFP18 has been described as the best near membrane indicator in that it shows much larger and faster transients compared to Fura-2.

Leakage resistant calcium indicators

Fura-2, Indo-1 and Fluo-3 tend to leak out of cells or accumulate in organelles. For some types of cells this leakage can be so great that it becomes difficult to obtain reliable measurements. This problem can be solved by using modified Ca^{2+}-indicators, like Fura-PE3 developed by Drs. Poenie and Minta (US patent 5,576,433). Leakage resistant modified indicators show the same spectral properties as the parent compounds.

New Mounting and Embedding Media for Microscopy

Fluka is pleased to offer an extended range of embedding media kits and ready-made mounting media for both light and electron microscopy. New non-fluorescent aqueous mounting media that delay fluorescence fading are now available. PVA-NPD is the medium of choice for AMCA, Cascade blue-labeled tissue sections and cell cultures. Either PVA-NPD or PVA-DABCO are recommended for FITC, TRITC, DTAF, Cy-3 labeled sections or cell cultures. Glycerol-Gelatin is another, well-known mounting medium for histochemistry that is compatible with fluorescent microscopy.

Fluka 49927	Glycerol-Gelatin
Fluka 10981	PVA-DABCO
Fluka 10979	PVA-NPD

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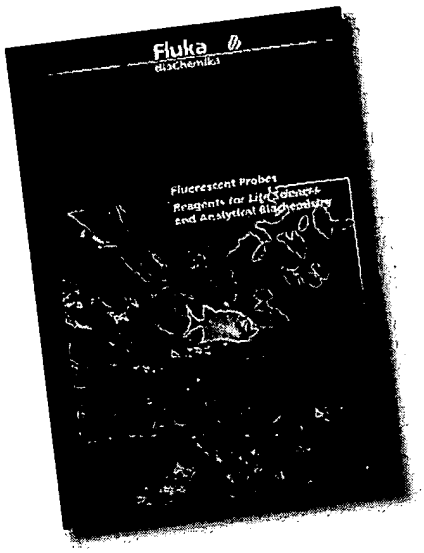
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Section 10.1 — Introduction to Enzyme Substrates and Their Reference Standards

Molecular Probes offers a large assortment of both common and uncommon fluorogenic and chromogenic enzyme substrates. We prepare substrates for enzyme-linked immunosorbent assays (ELISAs), as well as substrates for detecting very low levels of enzymatic activity in fixed cells, cell extracts and purified preparations. Our RediPlate product line includes enzyme substrates predispensed in 96-well or 384-well plates for high-throughput applications, along with the appropriate reference standards and other reaction components. We have also developed effective methods for detecting some enzymes in live cells. In this section, we describe the characteristics of our enzyme substrates and the fluorophores and chromophores from which they are derived, focusing primarily on the suitability of these substrates for different types of enzyme assays. The fluorophores and chromophores that are available as reference standards can be found in the data table and product list associated with this section. Substrates for specific enzymes are described in subsequent sections of this chapter.

Substrates Yielding Soluble Fluorescent Products

Solution assays designed to quantitate enzymatic activity in cell extracts or other biological fluids typically employ substrates that yield highly fluorescent or intensely absorbing, water-soluble products. ELISAs also rely on these substrates for indirect quantitation of analytes.[1–3] An ideal fluorogenic substrate for fluorescence-based solution assays yields a highly fluorescent, water-soluble product with optical properties significantly different from those of the substrate. If the fluorescence spectra of the substrate and product overlap significantly, analysis will likely require a separation step, especially when using excess substrate to obtain pseudo–first-order kinetics. Fortunately, many substrates have low intrinsic fluorescence or are metabolized to products that have longer-wavelength excitation or emission spectra (Figure 10.1). These fluorescent products can typically be quantitated in the presence of the unreacted substrate using a fluorometer or a fluorescence-based microplate reader. Microplate readers facilitate high-throughput analysis and require relatively small assay volumes, which usually reduces reagent costs. Moreover, the front-face optics in many microplate readers allows researchers to use more concentrated solutions, which may both improve the linearity of the kinetics and reduce inner-filter effects.

When the spectral characteristics of the substrate and its metabolic product are similar, techniques such as thin-layer chromatography (TLC), high-performance liquid chromatography (HPLC), capillary electrophoresis, solvent extraction or ion exchange can be used to separate the product from unconsumed substrate prior to analysis. For example, our *FAST* CAT Chloramphenicol Acetyltransferase Assay Kits (F-2900, F-6616, F-6617; Section 10.6) utilize chromatography to separate the intrinsically fluorescent substrates from their fluorescent products.

Substrates Derived from Water-Soluble Coumarins

Hydroxy- and amino-substituted coumarins have been the most widely used fluorophores for preparing fluorogenic substrates. Coumarin-based substrates produce highly soluble, intensely blue-fluorescent products. Phenolic dyes with high pK_as — such as 7-hydroxycoumarin (often called umbelliferone) and the more common 7-hydroxy-4-methylcoumarin (*β*-methylumbelliferone, H-189; Figure 10.2) — are not fully deprotonated and therefore not fully fluorescent unless the pH of the reaction mixture is raised to above pH ~10. Thus, substrates derived from these fluorophores are seldom used for continuous measurement of enzymatic activity in solution or live cells. The similar 3-cyano-7-hydroxycoumarin (C-183) and 6,8-difluoro-7-hydroxy-4-methylcoumarin

(DiFMU, D-6566; Figure 10.2, Figure 10.29) have lower pK_as [4] (Figure 1.101), making them suitable for a broader range of applications. Ether, ester and phosphate substrates derived from these phenolic dyes may be fluorescent but invariably exhibit shorter-wavelength absorption and emission spectra that can be easily distinguished from those of the product.

Aromatic amines, including the commonly used 7-amino-4-methylcoumarin (AMC, A-191; Figure 10.3), are partially protonated at low pH (less than ~5) but fully deprotonated at physiological pH. Thus, their fluorescence spectra are not subject to variability due to pH-dependent protonation/deprotonation when assayed near or above physiological pH. AMC is widely used to prepare peptidase substrates in which the amide has shorter-wavelength absorption and emission spectra than the amine hydrolysis product.

Substrates Derived from Water-Soluble Green to Yellow Fluorophores

Compared to coumarin-based substrates, substrates derived from fluoresceins, rhodamines, resorufins and some other dyes often provide significantly greater sensitivity in fluorescence-based enzyme assays. In addition, most of these longer-wavelength dyes have extinction coefficients that are five to 25 times that of coumarins, nitrophenols or nitroanilines, making them additionally useful as sensitive chromogenic substrates.

Hydrolytic substrates based on the derivatives of fluorescein (F-1300, Figure 1.54) or rhodamine 110 (R110, R-6479; Figure 10.4) usually incorporate two moieties, each of which serves as a substrate for the enzyme. Consequently, they are cleaved first to the monosubstituted analog and then to the free fluorophore. Because the monosubstituted analog often absorbs and emits light at the same wavelengths as the ultimate hydrolysis product, this initial hydrolysis complicates the interpretation of hydrolysis kinetics.[5] However, when highly purified, the disubstituted fluorescein- and rhodamine 110–based substrates have virtually no visible-wavelength absorbance or background fluorescence, making them extremely sensitive detection reagents. For example, researchers have reported that the activity of as few as 1.6 *molecules* of β-galactosidase can be detected with fluorescein di-β-D-galactopyranoside (FDG) and capillary electrophoresis.[6] Fluorogenic substrates based on either the AMC and R110 fluorophore are used in our EnzChek Caspase Assay Kits (Section 15.5) to detect apoptotic cells.

Chemical reduction of fluorescein- and rhodamine-based dyes yields colorless and nonfluorescent dihydrofluoresceins (Figure 19.6) and dihydrorhodamines (Figure 12.28). Although extremely useful for detection of reactive oxygen species (ROS) in phagocytic and other cells (Section 19.2), these dyes tend to be insufficiently stable for solution-based assays. An exception is our patented Amplex Gold reagent, which is utilized in our Amplex Gold and DyeChrome Double Western Blot Stain Kits (Figure 9.65). These kits are described in Section 9.4.

Substrates Derived from Water-Soluble Red Fluorophores

Long-wavelength fluorophores are often preferred because background absorbance and autofluorescence are generally lower when longer excitation wavelengths are used. Substrates derived from the red-fluorescent resorufin (R-363, Figure 10.5) and the dimethylacridinone derivative 7-hydroxy-9*H*-(1,3-dichloro-9,9-dimethylacridin-2-one) (DDAO, H-6482; Figure 10.6, Figure 10.11) contain only a single hydrolysis-sensitive moiety (Figure 10.27), thereby avoiding the biphasic kinetics of both fluorescein- and rhodamine-based substrates.[7]

Resorufin is used to prepare several substrates for glycosidases, hydrolytic enzymes and dealkylases. In most cases, the relatively low pK_a of resorufin (~6.0) permits continuous measurement of enzymatic activity. Thiols such as DTT or mercaptoethanol should be avoided in assays utilizing resorufin-based substrates. Our Amplex Red peroxidase substrate (A-12222, A-22177; Section 10.5) is a chemically reduced, colorless form of resorufin (Figure 10.49) that is oxidized back to resorufin by HRP in combination with hydrogen peroxide. Resorufin is also the product of enzyme-catalyzed reduction of resazurin (R-12204; Section 10.6, Section 15.2) — also

known as alamarBlue, a trademark of AccuMed International, Inc.

Substrates derived from DDAO, a red He–Ne laser–excitable fluorophore, generally exhibit good water solubility, low K_ms and high turnover rates.[8] In addition, the difference between the excitation maximum of the DDAO-based substrates and that of the product is greater than 150 nm (Figure 10.6), which allows the two species to be easily distinguished. We have utilized DDAO phosphate (D-6487, Section 10.3) in several of our Pro-Q Glycoprotein Blot Stain Kits, as well as in some of our DyeChrome and Pro-Q Western Blot Stain Kits (Section 9.4) for the sensitive detection of proteins. In our unique DyeChrome Double Western Blot Kit (D-21887, Section 9.4) we have combined the DDAO phosphate substrate with both the Amplex Gold HRP substrate and MPDF, a total-protein stain, for simultaneous trichromatic detection of two specific proteins and general proteins on Western blots (Figure 9.65).

Substrates for Live-Cell Enzyme Assays

Molecular Probes has developed a number of innovative strategies for investigating enzymatic activity in live cells.[9,10] For example, we offer a diverse set of probes that can passively enter the cell; once inside, they are processed by intracellular enzymes to generate products with improved cellular retention. We also offer kits and reagents for detecting the expression of several common reporter genes in cells and cell extracts. These include substrates for β-galactosidase (Section 10.2), β-glucuronidase (Section 10.2), secreted alkaline phosphatase (SEAP, Section 10.3), chloramphenicol acetyltransferase (CAT, Section 10.6) and luciferase (Section 10.6). Some of our EnzChek and DQ Kits are useful for study of the uptake and metabolism of proteins during phagocytosis (Section 16.1), as well as for the general screening of certain glycosidases (Section 10.2) and proteases (Section 10.4).

Thiol-Reactive Fluorophores

Molecular Probes prepares a number of enzyme substrates for live-cell assays that incorporate a mildly thiol-reactive chloromethyl moiety. Once inside the cell, this chloromethyl group undergoes what is believed to be a glutathione *S*-transferase–mediated reaction to produce a membrane-impermeant, glutathione–fluorescent dye adduct, although our experiments suggest that they may also react with other intracellular components. Regardless of the mechanism, many cell types loaded with these chloromethylated substrates are both fluorescent and viable for at least 24 hours after loading and often through several cell divisions. Furthermore, unlike the free dye, the peptide–fluorescent dye adducts contain amino groups and can therefore be covalently linked to surrounding biomolecules by fixation with formaldehyde or glutaraldehyde. This property permits long-term storage of the labeled cells or tissue and, in cases where the anti-dye antibody is available (Section 7.4), amplification of the conjugate by standard immunochemical techniques, including the tyramide signal-amplification (TSA, Section 6.2) and Enzyme-Labeled Fluorescence (ELF, Section 6.3) technologies. Chloromethyl analogs of fluorogenic substrates for glycosidases (our DetectaGene products, Section 10.2), peptidases, dealkylases, peroxidases and esterases are available. Our CellTracker Blue CMAC and CellTracker Blue CMF_2HC dyes (C-2110, Figure 14.8; C-12881, Figure 14.8) are precursors to peptidase and glycosidase substrates, respectively. They are also used for long-term cell tracing (Section 14.2). Molecular Probes' MitoTracker (Section 12.2) and CellTracker (Section 14.2) probes are also based on this principle.

Lipophilic Fluorophores

Lipophilic analogs of fluorescein and resorufin exhibit many of the same properties as the water-soluble fluorophores, including relatively high extinction coefficients and good quantum yields. In most cases, however, substrates based on these lipophilic analogs load more readily into cells, and their fluorescent products are better retained after cleavage than their water-soluble counterparts. Lipophilic substrates and their products probably also distribute differently in cells and may tend to associate with lipid regions of the cell. When passive cell loading or enhanced dye retention are critical parameters of the experiment, we recommend using our lipophilic substrates

for glycosidases (such as our ImaGene products, Section 10.2) and dealkylases (Section 10.6). Like resazurin (R-12204, Section 15.2), dodecylresazurin — the substrate in our Vybrant Cell Metabolic Assay Kit (V-23110, Section 15.3) — is reduced to dodecylresorufin by metabolically active cells; however, this lipophilic substrate is more useful than resazurin for microplate-based assays of all metabolic activity and permits single-cell analysis of cell metabolism (Figure 15.32) by flow cytometry and cell counting (Figure 15.33). Dodecylresorufin is also the product produced by hydrolysis of the β-galactosidase substrate (Figure 10.20) used in our ImaGene Red C_{12}RG lacZ Gene Expression Kit (I-2906, Section 10.2).

Pentafluorobenzoyl Fluorogenic Enzyme Substrates

Detecting enzymatic activity in live cells with fluorogenic substrates has been difficult both because the cell membrane is often a barrier to substrate penetration and because, once formed, the fluorescent product tends to leak from viable cells. We have found that our pentafluorobenzoyl (PFB) fluorogenic substrates address both of these difficulties.[11] First, when compared to conventional fluorescein-based substrates, several of our PFB substrates exhibit improved penetration through the cell membrane, permitting cell loading directly from culture medium. And second, the green-fluorescent PFB aminofluorescein (PFB-F, P-12925; Figure 10.7) released upon hydrolysis of the PFB-F substrates exhibits better cell retention than does fluorescein, the hydrolysis product of the fluorescein-based substrates. The hydrolysis products of the PFB substrates appear to be retained in viable cells by two mechanisms: 1) retention of the relatively lipophilic PFB group of the hydrolysis products in the cell membrane, and 2) glutathione *S*-transferase–catalyzed reaction of the hydrolysis products with intracellular glutathione.

Substrates Yielding Insoluble Fluorescent Products

Alkaline phosphatase, β-galactosidase and horseradish peroxidase (HRP) conjugates are widely used as secondary detection reagents for immunohistochemical analysis and *in situ* hybridization, as well as for protein and nucleic acid detection by Western, Southern and Northern blots. Also, various methods such as chromatography, isoelectric focusing and gel electrophoresis are commonly employed to separate enzymes preceding their detection. A review by Weder and Kaiser discusses the use of a wide variety of fluorogenic substrates for the detection of electrophoretically separated hydrolases.[12]

In order to precisely localize enzymatic activity in a tissue or cell, on a blot or in a gel, the substrate must yield a product that immediately precipitates or reacts at the site of enzymatic activity. In addition to the commonly used chromogenic substrates, including X-Gal, BCIP and NBT, Molecular Probes has developed fluorogenic ELF substrates for alkaline phosphatase and several other hydrolytic enzymes (Section 6.3). Our ELF substrates fluoresce only weakly in the blue range. However, upon enzymatic cleavage, these substrates form the intensely yellow-green–fluorescent ELF 97 alcohol (E-6578), which precipitates immediately at the site of enzymatic activity (Figure 6.23, Figure 7.10, Figure 10.8). The fluorescent ELF alcohol precipitate is exceptionally photostable (Figure 6.16) and has a high Stokes shift (Figure 6.17). We offer several ELF kits based on our ELF 97 phosphatase substrate; see Section 6.3 for a complete discussion of our ELF technology. The similar ELF 39 phosphate is used for detection of specific proteins in some of our DyeChrome Western Blot Stain Kits (Section 9.4, Figure 9.58). DDAO phosphate is very useful for solution-based assays but we have also been able to adapt it to yield a fluorescent precipitate that can detect proteins in Western blots; several kits containing DDAO phosphate are described in Section 9.4.

Tyramide signal-amplification (TSA) technology (Section 6.2) utilizes a unique concept in fluorescent substrates. Tyramide derivatives labeled with detectable moieties such as biotin or fluorophores are activated by HRP to a phenoxyl radical that is trapped near the site of its formation by reaction with nearby tyrosine residues (Figure 6.5). The covalent bond formed results in detection of HRP-labeled targets with high spatial resolution.

Substrates Based on Excited-State Energy Transfer

The principle of excited-state energy transfer (see "Fluorescence Resonance Energy Transfer (FRET)" in Section 1.3) can also be used to generate fluorogenic substrates. For example, the EDANS fluorophore in our HIV protease and renin substrates is effectively quenched by a nearby dabcyl acceptor chromophore (Figure 10.9). This chromophore has been carefully chosen for maximal overlap of its absorbance with the fluorophore's fluorescence, thus ensuring that the fluorescence is quenched through excited-state energy transfer. Proteolytic cleavage of the substrate results in spatial separation of the fluorophore and the acceptor chromophore, thereby restoring the fluorophore's fluorescence.[13–16] Many of the dyes described in Chapter 1 have been used to form energy-transfer pairs, some of which can be introduced during automated synthesis of peptides using modified amino acids described in Section 9.5. Table 1.9 lists our nonfluorescent quenching dyes and their spectral properties. Our QSY dyes (Section 1.6, Section 1.8) have spectral properties that are superior to those of the dabcyl chromophore (Table 1.10, Figure 1.73), making the QSY dyes useful as nonfluorescent quenchers for a broad range of fluorescent donor dyes (Figure 8.50).

The protease substrates in two of our EnzChek Protease Assay Kits and their RediPlate 96 and RediPlate 384 versions (Section 10.4) are heavily labeled casein conjugates; the close proximity of dye molecules results in considerable self-quenching. Hydrolysis of the protein to smaller fragments is accompanied by a dramatic increase in fluorescence, which forms the basis of a simple and sensitive continuous assay for a variety of proteases. In addition, we offer a phospholipase A substrate (bis-BODIPY FL C_{11}-PC, B-7701; Section 18.4) that contains a BODIPY FL fluorophore on each phospholipid acyl chain. Proximity of the BODIPY FL fluorophores on adjacent phospholipid acyl chains causes fluorescence self-quenching that is relieved only when the fluorophores are separated by phospholipase A–mediated cleavage. PED6, a phospholipid with a green-fluorescent BODIPY fatty acid on the lipid portion of the molecule and a 2,4-dinitrophenyl quencher on the polar head group (PED6, D-23739; Section 18.4; Figure 18.27) is useful as a specific phospholipase-A_2 substrate.[17]

Fluorescent Derivatization Reagents for Discontinuous Enzyme Assays

The mechanism of some enzymes makes it difficult to obtain a continuous optical change during reaction with an enzyme substrate. However, a discontinuous assay can often be developed by derivatizing the reaction products with one of the reagents described in Chapter 1, Chapter 2 and Chapter 3, usually followed by a separation step in order to generate a product-specific fluorescent signal. For example, fluorescamine (F-2332, F-20261; Section 1.8) or *o*-phthaldialdehyde (OPA, P-2331; Section 1.8) can detect the rate of *any* peptidase reaction by measuring the increase in the concentration of free amines in solution.[18,19] The activity of enzymes that produce free coenzyme A from its esters can be detected using thiol-reactive reagents such as 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB, D-8451; Section 5.2) or 7-fluorobenz-2-oxa-1,3-diazole-4-sulfonamide [20] (ABD-F, F-6053; Section 2.2). The products of enzymes that metabolize low molecular weight substrates can frequently be detected by chromatographic or electrophoretic analysis. HPLC or capillary zone electrophoresis can also be used to enhance the sensitivity of reactions that yield fluorescent products.[21] Measuring the activity of phospholipases, in particular, often requires chromatographic means to separate the detectable hydrolysis products (Section 18.4).

Substrates that Yield Insoluble Chromophoric Products

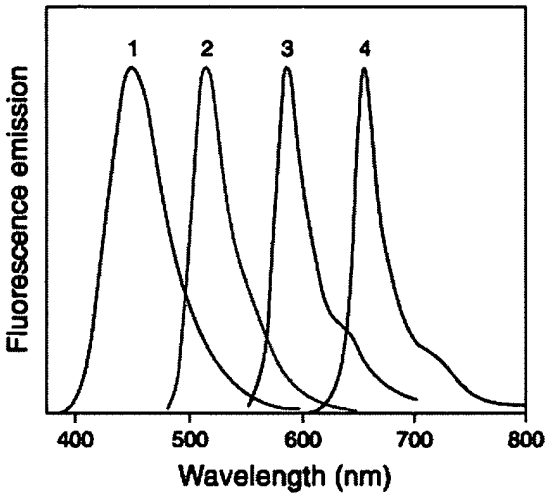
A number of chromogenic substrates for hydrolytic enzymes are derived from indolyl chromophores. These initially form a colorless — and sometimes blue-fluorescent — 3-hydroxyindole ("indoxyl"), which spontaneously, or through mediation of an oxidizing agent such as nitro blue tetrazolium (NBT, N-6495; Section 19.2) or potassium ferricyanide,[22] is converted to an intensely colored indigo dye that typically precipitates from the medium (Figure 9.64). Halogenated indolyl derivatives, including 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside

(X-Gal, B-1690, B-22015; Section 10.2) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP, B-6492, Section 10.3) are generally preferred because they produce finer precipitates that are less likely to diffuse from the site of formation, making them especially useful for detecting enzymatic activity in cells and tissues, on blots and in gels.

1. Methods Mol Biol 32, 461 (1994); **2.** J Immunol Methods 150, 5 (1992); **3.** Methods Enzymol 70, 419 (1980); **4.** Bioorg Med Chem Lett 8, 3107 (1998); **5.** Biochemistry 30, 8535 (1991); **6.** Anal Biochem 226, 147 (1995); **7.** Angew Chem Int Ed Engl 30, 1646 (1991); **8.** DDAO-based substrates are licensed to Molecular Probes under US 4,810,636 and its foreign equivalents; **9.** Biotech Histochem 70, 243 (1995); **10.** J Fluorescence 3, 119 (1993); **11.** US 5,773,236; **12.** J Chromatogr A 698, 181 (1995); **13.** Bioorg Med Chem Lett 2, 1665 (1992); **14.** J Protein Chem 9, 663 (1990); **15.** Science 247, 954 (1990); **16.** Tetrahedron Lett 31, 6493 (1990); **17.** Anal Biochem 276, 27 (1999); **18.** Biochemistry 29, 6670 (1990); **19.** Anal Biochem 123, 41 (1982); **20.** Chem Pharm Bull (Tokyo) 38, 2290 (1990); **21.** Anal Biochem 115, 177 (1981); **22.** Histochemie 23, 266 (1970).

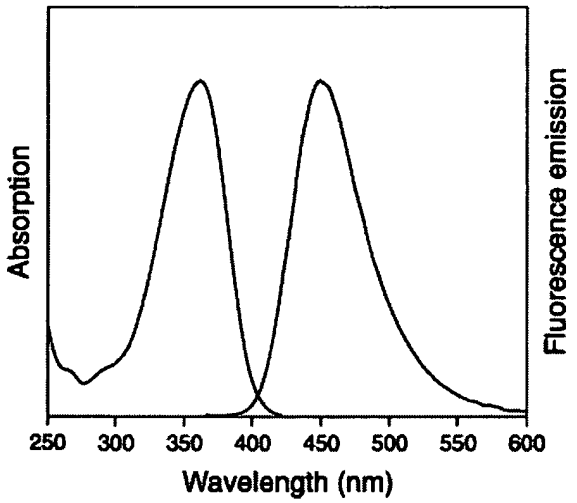
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Figure 10.1 Normalized emission spectra of **1)** 7-hydroxy-4-methylcoumarin (H-189) and 6,8-difluoro-7-hydroxy-4-methylcoumarin (DiFMU, D-6566), **2)** fluorescein (F-1300), **3)** resorufin (R-363) and **4)** DDAO (H-6482) in aqueous solution at pH 9. These fluorophores correspond to the hydrolysis or oxidation products of several of our fluorogenic enzyme substrates.



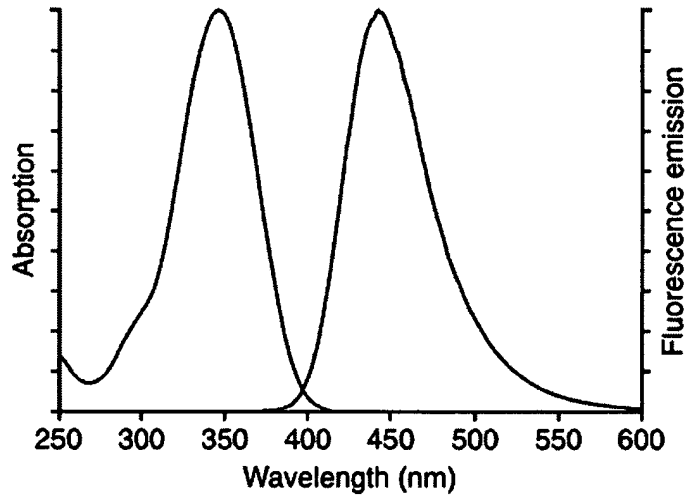
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Figure 10.2 Absorption and fluorescence emission spectra of 7-hydroxy-4-methylcoumarin (H-189) in pH 9.0 buffer. The spectra of 6,8-difluoro-7-hydroxy-4-methylcoumarin (D-6566) are essentially identical.



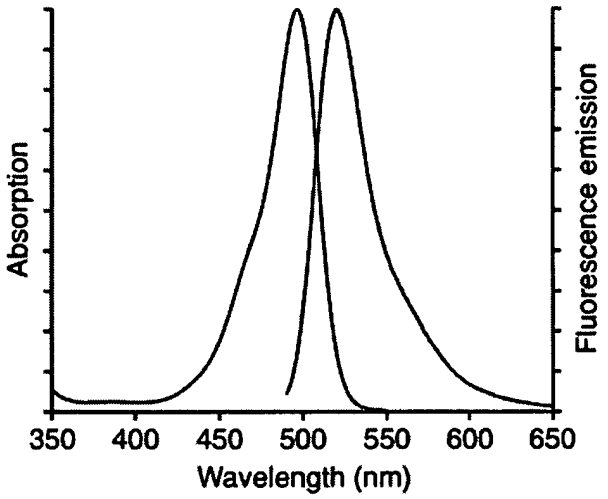
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Figure 10.3 Absorption and fluorescence emission spectra of 7-amino-4-methylcoumarin in pH 7.0 buffer.



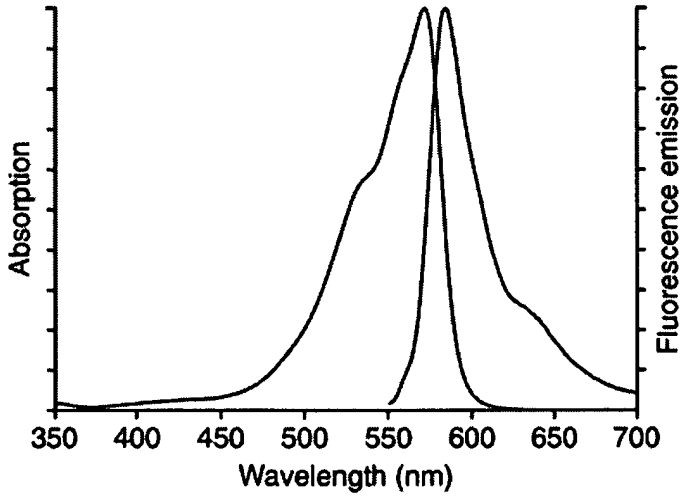
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Figure 10.4 Absorption and fluorescence emission spectra of rhodamine 110 in pH 7.0 buffer.



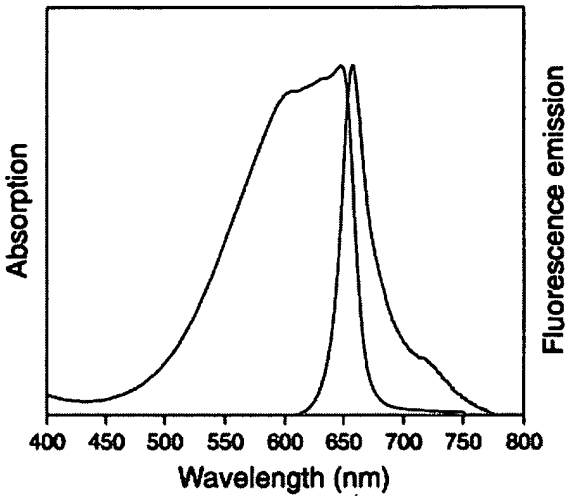
* * * * *

Figure 10.5 Absorption and fluorescence emission spectra of resorufin (R-363) in pH 9.0 buffer.



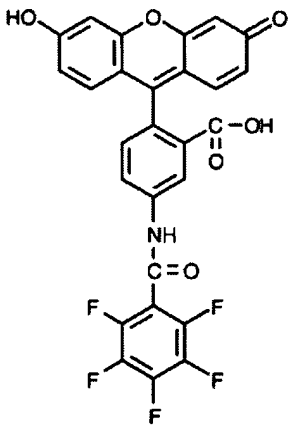
* * * * *

Figure 10.6 Normalized absorption and fluorescence emission spectra of DDAO, which is formed by alkaline phosphatase–mediated hydrolysis of DDAO phosphate (D-6487).



* * * * *

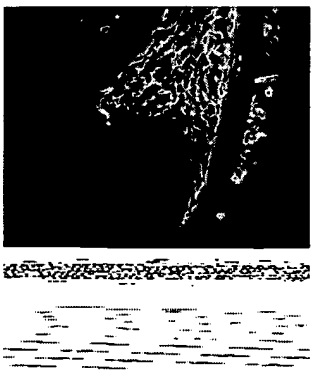
Figure 10.7 P-12925 5-(pentafluorobenzoylamino)fluorescein.



* * * * *

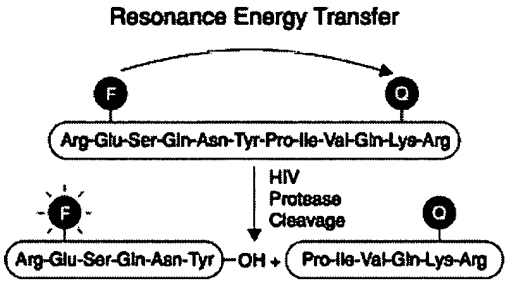
Figure 10.8 The yellow-green fluorescence of the ELF 97 alcohol precipitate, demonstrating localization of endogenous alkaline phosphatase activity in the ciliary body of the zebrafish eye. Adult zebrafish cryosections were first stained with Texas Red-X wheat germ agglutinin (W-21405) followed by the ELF 97 phosphate substrate in the ELF 97 Endogenous Phosphatase Detection Kit (E-6601), and finally the nuclei were counterstained with the blue-fluorescent Hoechst 33342 nucleic acid stain (H-1399, H-3570, H-21492). The triple-exposure image was acquired using bandpass filters appropriate for the Texas Red dye, ELF 97 alcohol precipitate and AMCA.





* * * * *

Figure 10.9 Principle of the fluorogenic response to protease cleavage exhibited by HIV protease substrate 1 (H-2930). Quenching of the EDANS fluorophore (F) by distance-dependent resonance energy transfer to the dabcyl quencher (Q) is eliminated upon cleavage of the intervening peptide linker.



* * * * *

Product List — 10.1 Introduction to Enzyme Substrates and Their Reference Standards

Cat #	Pr duct Name	Unit Size
A-191	7-amino-4-methylcoumarin *reference standard*	100 mg
C-2110	CellTracker™ Blue CMAC (7-amino-4-chloromethylcoumarin)	5 mg
C-12881	CellTracker™ Blue CMF_2HC (4-chloromethyl-6,8-difluoro-7-hydroxycoumarin)	5 mg
C-183	3-cyano-7-hydroxycoumarin	100 mg
D-6566	6,8-difluoro-7-hydroxy-4-methylcoumarin (DiFMU) *reference standard*	10 mg
E-6578	ELF® 97 alcohol *1 mM solution in DMSO*	1 mL
F-1300	fluorescein *reference standard*	1 g
H-6482	7-hydroxy-9*H*-(1,3-dichloro-9,9-dimethylacridin-2-one) (DDAO) *reference standard*	10 mg
H-189	7-hydroxy-4-methylcoumarin *reference standard*	1 g
P-12925	5-(pentafluorobenzoylamino)fluorescein (PFB-F)	5 mg
R-363	resorufin, sodium salt *reference standard*	100 mg
R-6479	rhodamine 110 (R110) *reference standard*	25 mg

* * * * *

Data Table — 10.1 Introduction to Enzyme Substrates and Their Reference Standards

Cat #	MW	Storage	Soluble	Abs	EC	Em	Solvent	Notes
A-191	175.19	L	DMF, DMSO	351	18,000	430	MeOH	1
C-183	187.15	L	pH >8, DMF	408	43,000	450	pH 9	
C-2110	209.63	F,D,L	DMSO	353	14,000	466	pH 9	2
C-12881	246.60	F,D,L	DMSO	371	16,000	464	pH 9	3
D-6566	212.15	L	DMSO, DMF	358	18,000	452	pH 9	3
E-6578	307.14	L	DMSO	345	ND	530	pH 8	4, 5, 6
F-1300	332.31	L	pH >6, DMF	490	88,000	514	pH 9	7
H-189	176.17	L	DMSO, MeOH	360	19,000	449	pH 9	3
H-6482	308.16	L	DMF	646	41,000	659	pH 10	
P-12925	541.39	F,D,L	DMSO, EtOH	492	80,000	516	pH 10	7
R-363	235.17	L	pH >7, DMF	571	54,000	585	pH 9	8, 9
R-6479	366.80	L	DMSO	499	92,000	521	MeOH	10
T-659	230.14	L	MeOH	385	16,000	502	pH 10	3

Notes

1. A-191 in aqueous solution (pH 7.0): Abs = 342 nm (EC = 16,000 $cm^{-1}M^{-1}$), Em = 441 nm.
2. C-2110 in MeOH: Abs = 364 nm (EC = 16,000 $cm^{-1}M^{-1}$), Em = 454 nm.
3. Spectra of hydroxycoumarins are pH-dependent. Below the pK_a, Abs shifts to shorter wavelengths (325–340 nm) and fluorescence intensity decreases. Approximate pK_a values are 7.8 (H-189, C-2111), 7.5 (H-185), 7.3 (T-659) and 4.9 (D-6566, C-12881).
4. ND = not determined.
5. This product is supplied as a ready-made solution in the solvent indicated under "Soluble."
6. ELF 97 alcohol is insoluble in water. Spectral maxima listed are for an aqueous suspension; for this reason, the value of EC cannot be determined.
7. Absorption and fluorescence of fluorescein derivatives are pH-dependent. Extinction coefficients and fluorescence quantum yields decrease markedly at pH <7.
8. Absorption and fluorescence of resorufin are pH-dependent. Below the pK_a (~6.0), Abs shifts to ~480 nm and both EC and the fluorescence quantum yield are markedly lower.
9. R-363 is unstable in the presence of thiols such as dithiothreitol (DTT) and 2-mercaptoethanol.
10. R-6479 in aqueous solution (pH 7.0): Abs = 496 nm (EC = 83,000 $cm^{-1}M^{-1}$), Em = 520 nm.

* * * * *

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Section 10.2 — Detecting Glycosidases

Glycosidase enzymes exhibit very high selectivity for hydrolysis of their preferred sugars. For example, β-galactosidase rapidly hydrolyzes β-D-galactopyranosides but usually does not hydrolyze either the anomeric α-D-galactopyranosides or the isomeric β-D-glucopyranosides. Endogenous glycosidase activity is frequently used to characterize strains of microorganisms [1–3] and to selectively label organelles of mammalian cells;[4–6] defects in glycosidase activity are characteristic of several diseases.[7–9]

In addition, glycosidases are important reporter gene markers. Specifically, *lacZ*, which encodes β-galactosidase, is extensively used as a reporter gene in animals and yeast, whereas the β-glucuronidase (GUS) gene is a popular reporter gene in plants.[10–13] Glycosidase substrates are also used in conjunction with glycosidase-conjugated secondary detection reagents in immunohistochemical techniques and enzyme-linked immunosorbent assays [14] (ELISAs). Molecular Probes' complete line of fluorogenic and chromogenic glycosidase substrates is listed in Table 10.1.

Some General Fluorogenic β-Galactosidase Substrates

Flu rescein Digalactoside

Probably the most sensitive fluorogenic substrate for detecting β-galactosidase is fluorescein di-β-D-galactopyranoside [15] (FDG, F-1179; Figure 10.10). Nonfluorescent FDG is sequentially hydrolyzed by β-galactosidase, first to fluorescein monogalactoside (FMG) and then to highly fluorescent fluorescein (F-1300, Section 10.1, Figure 1.54). Enzyme-mediated hydrolysis of FDG can be followed by the increase in either absorbance or fluorescence. Although the turnover rates of FDG and its analogs are considerably slower than that of the common spectrophotometric galactosidase substrate, 2-nitrophenyl β-D-galactopyranoside (ONPG),[16,17] the absorbance of fluorescein is about fivefold greater than that of *o*-nitrophenol. Moreover, fluorescence-based measurements can be several orders of magnitude more sensitive than absorbance-based measurements. Fluorescence-based assays employing FDG are also reported to be 100- to 1000-fold more sensitive than radioisotope-based ELISAs.[18]

In addition to its use in ELISAs, the FDG substrate has proven very effective for identifying *lacZ*-positive cells with fluorescence microscopy [19–22] and flow cytometry.[23–28] FDG has been employed to identify cells infected with recombinant herpesvirus,[29] to detect unique patterns of β-galactosidase expression in live transgenic zebrafish embryos [19] and to monitor β-galactosidase expression in bacteria.[20,30,31] The purity of FDG and its analogs is very important because a reagent with extremely low fluorescence background is essential for most applications. Our stringent quality control ensures that the fluorescent contamination of FDG is less than 50 ppm.

The FluoReporter *lacZ* Flow Cytometry Kits (50-test kit, F-1930; 250-test kit, F-1931) provide materials and protocols for quantitating β-galactosidase activity with FDG in single cells using flow cytometry. These kits are accompanied by a license to practice patented techniques for loading FDG by hypotonic shock and improving retention of fluorescein in *lacZ*-positive cells.[32] In addition to a detailed protocol, each FluoReporter *lacZ* Flow Cytometry Kit contains convenient premixed solutions of:

- FDG
- Phenylethyl β-D-thiogalactopyranoside (PETG; also available separately as a solid, P-1692), a broad-spectrum β-galactosidase inhibitor for stopping the reaction [33]

- Chloroquine diphosphate for inhibiting hydrolysis of the substrate in acidic organelles by endogenous galactosidase enzymes
- Propidium iodide for detecting dead cells

A sufficient amount of each reagent is provided in these kits for 50 (in Kit F-1930) or 250 (in Kit F-1931) flow cytometry assays. This assay enables researchers to detect heterogeneous expression patterns and to sort and clone individual cells expressing known quantities of β-galactosidase. Practical reviews on using FDG for flow cytometric analysis and sorting of *lacZ*-positive cells are available.[34,35]

FDG's fluorescent hydrolysis product, fluorescein (F-1300, Section 10.1, Figure 15.3), rapidly leaks from cells under physiological conditions, making FDG's use problematic for prolonged studies. Our DetectaGene and ImaGene substrates (see below) have been specifically designed to improve retention of the fluorescent products in cells.

Resorufin Galactoside

Unlike FDG, resorufin β-D-galactopyranoside (R-1159) requires only a single-step hydrolysis reaction to attain full fluorescence.[36] This substrate is especially useful for sensitive enzyme measurements in ELISAs.[14,37] The relatively low pK_a (~6.0) of its hydrolysis product, resorufin (R-363, Section 10.1, Figure 10.5), permits its use for continuous measurement of enzymatic activity at physiological pH. Resorufin galactoside has also been used to quantitate β-galactosidase activity in single yeast cells by flow cytometry [38] and to detect immobilized β-galactosidase activity in bioreactors.[39,40]

DDAO Galactoside

Although 7-hydroxy-9*H*-(1,3-dichloro-9,9-dimethylacridin-2-one) (DDAO)-based substrates are intrinsically fluorescent (excitation/emission ~460/610 nm), β-galactosidase–catalyzed hydrolysis of DDAO galactoside (D-6488) liberates the DDAO fluorophore (Figure 10.11), which absorbs and emits light at much longer wavelengths (excitation/emission ~645/660 nm) [41] (Figure 10.12). Not only can DDAO (H-6482, Section 10.1) be excited without interference from the substrate, but its fluorescence emission is detected at wavelengths that are well beyond the autofluorescence exhibited by most biological samples. The relatively low pK_a of DDAO (~5.5) permits continuous monitoring of β-galactosidase activity at physiological pH.

Methylumbelliferyl Galactoside and Its Difluorinated Analog

The fluorogenic β-galactosidase substrate β-methylumbelliferyl β-D-galactopyranoside (MUG, M-1489) is commonly used to detect β-galactosidase activity in cell extracts,[42–44] lysosomes [45] and human blood serum.[46] However, MUG's hydrolysis product, 7-hydroxy-4-methylcoumarin (β-methylumbelliferone, H-189; Section 10.1; Figure 10.2), has a relatively high pK_a (~7.8), precluding its use for continuous measurement of enzymatic activity.
6,8-Difluoro-4-methylumbelliferyl β-D-galactopyranoside (DiFMUG, D-11945) yields a hydrolysis product — 6,8-difluoro-7-hydroxy-4-methylcoumarin (D-6566, Section 10.1, Figure 10.2) — with a much lower pK_a (4.9),[47] allowing its detection at physiological pH.[48,49] Given the very low pK_a of its hydrolysis product, DiFMUG should be especially useful for the continuous *in vitro* assay of β-D-galactosidase activity at any pH >6. The fluorinated coumarin glycosides are patented by Molecular Probes.

Carbo xyumbelliferyl Galacto side and the Fluo Repo rter *lacZ*/Galacto sidase Quantitati on Kit

Hydrolysis of 3-carboxyumbelliferyl β-D-galactopyranoside (CUG, C-1488) by β-galactosidase yields 7-hydroxycoumarin-3-carboxylic acid (H-185, Section 10.1). 7-Hydroxycoumarin has a pK_a below the pH at which the turnover rate is optimal, facilitating the use of CUG for continuous measurements of β-galactosidase activity. Unlike most substrates for β-galactosidase, CUG is quite water-soluble and can be used over a wide range of concentrations in enzymatic activity measurements.[50–52] Our FluoReporter *lacZ*/Galactosidase Quantitation Kit (F-2905) provides a CUG-based method for quantitating β-galactosidase activity in ELISAs or *lacZ*-positive cell extracts. Each kit contains:

- CUG
- 7-Hydroxycoumarin-3-carboxylic acid, a reference standard
- A detailed protocol suitable for use with any fluorescence-based microplate reader

Sufficient reagents are provided for approximately 1000 β-galactosidase assays. We have demonstrated a practical detection limit of ~0.5 pg of β-galactosidase using this kit and a fluorescence microplate reader.

Flu rescent Glycosphingolipids

β-Galactosidase enzymes that act on the lipophilic sphingosyl galactosides, including galactosylceramidase (EC 3.2.1.46) and G_{M1} ganglioside β-galactosidase (EC 3.2.1.23), are particularly important in neurochemistry. The preferred substrates for these enzymes are sphingolipids derived from galactose (Section 13.3). Galactosylceramidase converts substrates such as our BODIPY FL C_{12}-galactosylceramide (D-7519) back to the ceramide. Purified G_{M1} ganglioside galactosidase removes the terminal galactose residue from lactosylceramides such as our BODIPY FL C_5-lactosylceramide [53] (D-13951), yielding the corresponding glucosylceramide.[54] However, the lack of a spectral shift of the hydrolysis products means that extraction and chromatographic separation of the products is necessary for assessment of the activity.

Amplex Red Galactose/Galactose Oxidase Assay Kit

Our Amplex Red reagent (10-acetyl-3,7-dihydroxyphenoxazine, A-12222, A-22177; Section 10.5; Figure 10.49) is an unusually stable peroxidase substrate that we have used in coupled reactions to detect a wide variety of analytes, including both enzymes and their substrates (see Section 10.5 for a list of all of our Amplex Red Kits and reagents). Most of the assays can be performed as continuous assays at neutral or slightly acidic pH and are particularly suitable for automation and high-throughput screening using either an absorption- or fluorescence-based microplate reader.

Rather than requiring an unnatural fluorogenic or chromogenic substrate for β-galactosidase (or α-galactosidase), our Amplex Red reagent–based technology permits the direct quantitation of free galactose, which is produced by a wide variety of enzymes. Even enzymes that act on polysaccharides and glycolipids that cannot be easily assayed with any known chromogenic substrate can, in some cases, be detected and their activity quantitated using the Amplex Red reagent in combination with galactose oxidase and horseradish peroxidase. Unlike glucose oxidase, galactose oxidase can produce H_2O_2 from either free galactose or from polysaccharides — including glycoproteins in solution and on cell surfaces — and from certain glycolipids in which galactose is the terminal residue (Figure 10.13). Because the galactose oxidase–catalyzed reaction does not require prior cleavage of the glycoside to free galactose by a galactosidase, appropriate control reactions must be used to ascertain whether the rate-limiting step is the galactosidase- or galactose oxidase–mediated reaction.

The Amplex Red Galactose/Galactose Oxidase Assay Kit (A-22179) provides an ultrasensitive method for detecting galactose (Figure 10.14) and galactose oxidase (Figure 10.15) activity. This assay utilizes the Amplex Red reagent (Figure 10.49) to detect H_2O_2 generated by galactose

oxidase–mediated oxidation of desialated galactose moieties. In the presence of horseradish peroxidase (HRP), the H_2O_2 thus produced reacts with the Amplex Red reagent in a 1:1 stoichiometry to generate the red-fluorescent oxidation product, resorufin.[55] Resorufin has absorption and fluorescence emission maxima of approximately 563 nm and 587 nm, respectively (Figure 10.5), and because its extinction coefficient is high (54,000 $cm^{-1}M^{-1}$), the assay can be performed either fluorometrically or spectrophotometrically. The Amplex Red Galactose/Galactose Oxidase Assay Kit provides all the reagents and a general protocol for the assay of galactose-producing enzymes or for the assay of galactose oxidase, including:

- The Amplex Red reagent
- Dimethylsulfoxide (DMSO)
- D-Galactose
- Galactose oxidase from *Dactylium dendroides*
- Horseradish peroxidase (HRP)
- Hydrogen peroxide (H_2O_2)
- A 5x reaction buffer
- A detailed protocol for the assay

Sufficient reagents are provided for approximately 400 assays using either an absorption- or fluorescence-based microplate reader and reaction volumes of 100 µL per assay. The Amplex Red galactose/galactose oxidase assay accurately measures as low as 4 µM galactose and 2 mU/mL galactose oxidase activity (Figure 10.14, Figure 10.15). Because of the high absorbance of resorufin, the absorptimetric assay has only slightly lower sensitivity than the fluorometric assay.

Modified Fluorogenic β-Galactosidase Substrates with Improved Cellular Retention

The primary problems associated with detecting *lacZ* expression in live cells using fluorogenic substrates are:

- Difficulty in loading the substrates under physiological conditions
- Leakage of the fluorescent product from live cells
- High levels of endogenous β-galactosidase activity in many cells

Our DetectaGene and ImaGene Kits are designed to improve the sensitivity of β-galactosidase assays by yielding products that are better retained in viable cells and, in the case of the ImaGene Kits, by providing substrates that can be passively loaded into live cells. The high level of endogenous β-galactosidase activity remains an obstacle when detecting low levels of *lacZ* expression.

DetectaGene Green *lacZ* Gene Expression Kit

The substrate in our DetectaGene Green *lacZ* Gene Expression Kit (D-2920) — 5-chloromethylfluorescein di-β-D-galactopyranoside (CMFDG) — is a galactose derivative that has been chemically modified to include a mildly thiol-reactive chloromethyl group (Figure 10.16). Once loaded into the cell using the Influx pinocytic cell-loading reagent (I-14402; included in Kit D-2920) or by microinjection, hypotonic shock or another technique (Table 14.1), the DetectaGene substrate undergoes two reactions: 1) its two galactose moieties are cleaved by intracellular β-galactosidase and 2) either simultaneously or sequentially, its chloromethyl moiety reacts with glutathione and possibly other intracellular thiols to form a membrane-impermeant, peptide–fluorescent dye adduct [56] (Figure 10.16). Because peptides do not readily cross the plasma membrane, the resulting fluorescent adduct is much better retained than is the free dye, even in cells that have been kept at 37°C. We have found that *lacZ*-positive cells loaded from medium containing 1 mM CMFDG are as fluorescent as those loaded with 40-fold higher concentrations of FDG. Furthermore, unlike the free dye, the peptide–fluorescent dye adducts contain amino groups

and can therefore be covalently linked to surrounding biomolecules by fixation with formaldehyde or glutaraldehyde. This property permits long-term storage of the labeled cells or tissue, as well as amplification of the conjugate by standard immunohistochemical techniques using anti-fluorescein antibody (Section 7.4, Table 7.16).

The CMFDG substrate in our DetectaGene Green *lacZ* Gene Expression Kit was used to stain *lacZ*-expressing floor plate cells in tissue dissected from a developing mouse embryo,[57] to identify *lacZ*-enhancer–trapped *Drosophila* neurons in culture and to detect β-galactosidase activity in hippocampal slices.[58] In the latter study, the fluorescence of the neurons could still be visualized 24 hours after dye loading, and the fluorescent CMFDG-loaded neurons exhibited a normal pattern and time course of axonal outgrowth and branching.[59] CMFDG also has been microinjected into primary hepatocytes, fibroblasts and glioma cells to detect β-galactosidase activity [60] and has been incorporated into an electrophysiological recording pipette to confirm the identity of neurons cotransfected with the *lacZ* gene and a second gene encoding Ca^{2+}/calmodulin-dependent protein kinase II (CaM kinase II).[61]

The DetectaGene Green CMFDG *lacZ* Gene Expression Kit (D-2920) contains:

- The DetectaGene Green CMFDG substrate (Figure 10.16)
- Phenylethyl β-D-thiogalactopyranoside (PETG; also available separately as a solid, P-1692), a broad-spectrum β-galactosidase inhibitor for stopping the reaction [33]
- Verapamil for inhibiting product efflux [62,63]
- Chloroquine diphosphate for inhibiting acidic hydrolysis of the substrate
- Propidium iodide for detecting dead cells
- Influx pinocytic cell-loading reagent for introducing CMFDG into cells
- A detailed protocol for detecting β-galactosidase activity

When used at the recommended dilutions, a sufficient amount of each reagent is provided for approximately 200 flow cytometry tests with the DetectaGene Green CMFDG Kit. Verapamil has been added to the DetectaGene Green CMFDG *lacZ* Gene Expression Kit because we have observed that cell retention of the fluorescent dye–peptide adduct can be considerably improved in many cell types by adding verapamil to the medium.[62]

PFB Aminofluorescein Digalactoside

Our patented 5-(pentafluorobenzoylamino)fluorescein di-β-D-galactopyranoside (PFB-FDG, P-11948; Figure 10.17) yields the green-fluorescent PFB-F dye (P-12925, Section 10.1), which appears to localize to endosomal and lysosomal compartments when loaded into cells by pinocytosis (Figure 10.18), similar to our PFB aminofluorescein diglucoside (PFB-FDGlu, P-11947 see below). Thus, PFB-FDG [64] is potentially useful for studying lysosomal storage diseases, including Krabbe's disease, G_{M1} gangliosidosis, galactosialidosis and Morquio's syndrome, type B, which are all associated with deficient lysosomal β-galactosidase activity.[65]

ImaGene *lacZ* Reagents and Gene Expression Kits

The fluorescein- and resorufin-based galactosidase substrates in our ImaGene Green and ImaGene Red *lacZ* Gene Expression Kits (I-2904, I-2906) have been covalently modified to include a 12-carbon lipophilic moiety. Unlike FDG or CMFDG (Figure 10.16), these lipophilic fluorescein- and resorufin-based substrates — abbreviated C_{12}FDG (Figure 10.19) and C_{12}RG (Figure 10.20) for the ImaGene Green and ImaGene Red substrates, respectively — can be loaded simply by adding the substrate to the aqueous medium in which the cells or organisms are growing, either at ambient temperatures or at 37°C. Once inside the cell, the substrates are cleaved by β-galactosidase, producing fluorescent products that are well-retained by the cells, probably by incorporation of their lipophilic tails within the cellular membranes. Mammalian NIH 3T3 *lacZ*-positive cells grown

for several days in medium containing 60 µM C_{12}FDG appear morphologically normal, continue to undergo cell division and remain fluorescent for up to three cell divisions after replacement with substrate-free medium.[56,66]

The C_{12}FDG substrate in our ImaGene Green *lacZ* Expression Kit (I-2904) is superior to FDG for flow cytometric detection of β-galactosidase activity in live mammalian cells.[67] Using C_{12}FDG with flow cytometric methods, researchers have:

- Assessed levels of *lacZ* gene expression in recombinant Chinese hamster ovary (CHO) cells throughout the cell cycle, which was monitored with Hoechst 33342 [68] (H-1399; H-3570; FluoroPure Grade, H-21492; Section 8.1)
- Identified endocrine cell precursors in dissociated fetal pancreatic tissue based on their high levels of endogenous acid β-galactosidase [69]
- Measured β-galactosidase activity in single recombinant *E. coli* bacteria [70]
- Detected the activity of β-galactosidase fusion proteins in yeast [71]
- Sorted β-galactosidase–expressing mouse sperm cells [72] and insect cells that harbor recombinant baculovirus [73,74]

The C_{12}FDG substrate was also useful in a fluorescence microscopy study of zebrafish expressing a *lacZ* reporter gene that was under the control of a mammalian homeobox gene promoter.[75] In addition, lipophilic β-galactosidase substrates have been employed to diagnose the deficiency in β-galactocerebrosidase activity that typifies Krabbe's disease in human patients.[8,76] In some cell types, C_{12}FDG produces high levels of background fluorescence that may prohibit its use in assaying low β-galactosidase expression.[21]

Molecular Probes' ImaGene Green C_{12}FDG or ImaGene Red C_{12}RG *lacZ* Gene Expression Kits contain:

- ImaGene Green C_{12}FDG (in Kit I-2904) *or* ImaGene Red C_{12}RG (in Kit I-2906)
- Phenylethyl β-D-thiogalactopyranoside (PETG; also available separately as a solid, P-1692), a broad-spectrum β-galactosidase inhibitor for stopping the reaction [33]
- Chloroquine diphosphate for inhibiting acidic hydrolysis of the substrate
- A detailed protocol for detecting β-galactosidase activity

A sufficient amount of each reagent is provided for 100–200 assays, depending on the volume used for each experiment.

5-Dodecanoylaminofluorescein di-β-D-galactopyranoside (C_{12}FDG) is available separately (D-2893) and we also offer the somewhat less lipophilic 5-octanoylaminofluorescein di-β-D-galactopyranoside (C_8FDG, O-2892). The C_8FDG analog is optimal for investigating the expression of *lacZ* fusion genes in sporulating cultures of *Bacillus subtilis* [77] and is a better substrate than C_{12}FDG for the detection of β-galactosidase activity in sperm containing the *lacZ* gene.[78] 5-Acetylaminofluorescein di-β-D-galactopyranoside (C_2FDG, A-22010) is particularly useful for detecting *lacZ* reporter gene expression in slow-growing mycobacteria, including *Mycobacterium tuberculosis*, using a fluorescence plate reader or a flow cytometer.[79] The ImaGene substrates in these kits are patented by Molecular Probes.[80]

Chloromethyl and Lip philic Derivatives f DiFMUG

The relatively low pK_a of our 6,8-difluoro-7-hydroxycoumarin derivatives (Figure 1.101) has also allowed us to develop some useful probes for detecting enzymatic activity *in vivo*. Although the β-galactosidase DiFMUG (D-11945) readily enters many live eukaryotic cells, its hydrolysis product (6,8-difluoro-7-hydroxy-4-methylcoumarin, DiFMU; D-6566; Section 10.1; Figure 10.29) is not well retained. To address this limitation, we have developed two modified galactosidase substrates using product-retention strategies that have proven useful for our patented DetectaGene and ImaGene glycosidase substrates.

As with our DetectaGene products, we have replaced the methyl group of DiFMUG with a mildly thiol-reactive chloromethyl group, yielding the β-galactosidase substrate CMDiFUG (C-11946), which is the 6,8-difluorinated analog of 4-chloromethylcoumarin-7-yl-β-D-galactopyranoside (CMCG). We have previously shown that incorporating a chloromethyl group into dyes, as in our CellTracker and MitoTracker probes,[63,81] considerably improves the retention of fluorescent products in live cells. This enhanced cell retention is at least partially attributable to the formation of dye conjugates with intracellular thiols, including glutathione.[82] Our results indicate that CMDiFUG discriminates *lacZ*-positive and *lacZ*-negative live cells better than all the other fluorogenic β-galactosidase substrates we have tested, including our ImaGene and DetectaGene substrates.

Similar to our ImaGene products, 6,8-difluoro-4-heptadecylumbelliferyl β-D-galactopyranoside (C_{17}DiFUG, D-11950) contains a lipophilic moiety in place of the methyl group of DiFMUG (Figure 10.21). This modification improves the penetration of this substrate through cell membranes, as well as the retention of the fluorescent product of β-galactosidase activity in live cells. The fluorinated coumarin glycosides are patented by Molecular Probes.

Fluorogenic β-Glucuronidase Substrates

The substrate 4-methylumbelliferyl β-D-glucuronide (MUGlcU, M-1490) is probably the most commonly used fluorogenic reagent for identifying *E. coli* contamination and for detecting GUS reporter gene expression in plants and plant extracts.[83,84] However, β-glucuronidase substrates based on fluorescein may be much more sensitive and yield products that are fluorescent at physiological pH, making them useful for continuous monitoring of enzymatic activity. In addition, we offer a fluorogenic ELF 97 β-D-glucuronidase substrate (E-6587), which produces an intensely green-fluorescent precipitate at the site of enzymatic activity that can be clearly distinguished from most autofluorescence.[85] This substrate has been used for in-gel zymography to detect β-glucuronidase activity [86–88] (Figure 9.24, Figure 10.22), immunoassays on protein microarrays [89] and for the flow cytometric analysis and separation of *E. coli* that had been transfected with *gusA* expression vectors.[90]

Fluorescein Diglucuronide

Fluorescein di-β-D-glucuronide (FDGlcU, F-2915) is colorless and nonfluorescent until it is hydrolyzed to the monoglucuronide and then to highly fluorescent fluorescein (F-1300, Section 10.1). FDGlcU has been used to detect β-glucuronidase activity in plant extracts containing the GUS reporter gene [84] and may also be useful for assaying lysosomal enzyme release from neutrophils.[91,92] FDGlcU has also been used in the flow cytometric assay of individual mammalian cells expressing the *E. coli* β-glucuronidase gene.[93]

PFB Aminofluorescein Diglucuronide

Our patented 5-(pentafluorobenzoylamino)fluorescein di-β-D-glucuronide (PFB-FDGlcU, P-11949) yields the green-fluorescent PFB-F (P-12925, Section 10.1), which appears to localize to endosomal and lysosomal compartments when loaded into cells by pinocytosis, similar to our PFB aminofluorescein diglucoside (PFB-FDGlu, P-11947). PFB-FDGlcU has been used for the quantitative

analysis of β-glucuronidase activity in viable cells and for sorting high-expressing cells by flow cytometry.[94] Enzyme enrichment has promise as a tool for gene therapy.

Coumarin Glucuronides

4-Methylumbelliferyl β-D-glucuronide (MUGlcU, M-1490) has been used extensively to detect *E. coli* in food,[95,96] water,[97] urine [98] and environmental samples.[99] MUGlcU is stable to the conditions required for sterilization of media. A fluorogenic bioassay using MUGlcU has been developed to assess the detrimental effects of Li^+, Al^{3+}, Cr^{6+} and Hg^{2+} on the proliferation of *E. coli*.[100]

MUGlcU is also commonly used to identify plant tissue expressing the GUS reporter gene,[101–103] including nondestructive assays that allow propagation of the transformed plant lines.[88,104] In addition, MUGlcU has served as a sensitive substrate for lysosomal enzyme release from neutrophils.[91,92]

Enzyme-mediated hydrolysis of 6,8-difluoro-4-methylumbelliferyl β-D-glucuronide (DiFMUGlcU, D-11951) yields a highly fluorescent product (6,8-difluoro-7-hydroxy-4-methylcoumarin, DiFMU; D-6566; Section 10.1; Figure 10.29) that has a very low pK_a, which should make DiFMUGlcU especially useful for the continuous *in vitro* assay of β-D-glucuronidase activity at a pH greater than or equal to 6. The hydrolysis product of β-trifluoromethylumbelliferyl β-D-glucuronide (T-658) exhibits longer-wavelength excitation and emission spectra than those of either MUGlcU or DiFMUGlcU, which can be advantageous for cells that have high levels of endogenous fluorescence, such as plant cells.

ImaGene Green β-D-Glucuronidase Substrate

Molecular Probes also offers a lipophilic analog of fluorescein di-β-D-glucuronide in our ImaGene Green C_{12}FDGlcU GUS Gene Expression Kit (I-2908). As with our similar ImaGene substrates for β-galactosidase (see above), we have shown that this lipophilic β-glucuronidase substrate freely diffuses across the membranes of viable cultured tobacco leaf cells or protoplasts under physiological conditions. Furthermore, the fluorescent cleavage product is retained in the plant cell for hours to days, facilitating long-term measurements of GUS gene expression. In thin sections of transgenic tomato leaf, the ImaGene Green C_{12}FDGlcU GUS Gene Expression Kit provided a simple and reliable GUS assay that, coupled with confocal laser-scanning microscopy, yielded good cellular resolution.[105] The substrate has also been used to detect β-glucuronidase activity in an *Acremonium* transformant containing the GUS reporter gene.[106]

Molecular Probes' ImaGene Green C_{12}FDGlcU GUS Gene Expression Kit contains:

- ImaGene Green C_{12}FDGlcU
- D-Glucaric acid-1,4-lactone, a β-glucuronidase inhibitor for stopping the reaction
- A detailed protocol for detecting β-glucuronidase activity

A sufficient amount of each reagent is provided for approximately 100 tests, depending on the volume used for each experiment.

ELF 97 β-D-Glucuronide

Molecular Probes' ELF 97 β-D-glucuronidase substrate [107] (ELF 97 β-D-glucuronide, E-6587) may be the ideal substrate for analyzing GUS enzyme activity in transgenic plants. Upon hydrolysis, this fluorogenic substrate produces a bright yellow-green–fluorescent precipitate at the site of enzymatic activity. This fluorescent precipitate has some unique spectral characteristics, including an extremely large Stokes shift (Figure 6.17), that make it easily distinguishable from the

endogenous fluorescent components commonly found in plants (see Section 6.3 for a description of our patented ELF technology). We have used this substrate to detect the GUS enzyme in *Arabidopsis* and have found that, after only four hours incubation, signal can be detected in whole-leaf cuttings from GUS-positive plants.[88] Homogenization of a small portion of a leaf from a GUS-positive *Arabidopsis* plant followed by separation on a nondenaturing gel yields a discrete band corresponding to the glucuronidase enzyme (Figure 10.22). We have also used the ELF 97 glucuronidase substrate for in-gel zymography in one aspect of our Mutiplexed Proteomics technology (Section 9.4, Figure 9.24). It is possible to detect as little as 0.5 ng of purified β-glucuronidase in a nondenaturing gel incubated with ELF 97 glucuronide. This substrate may also be useful for detecting GUS fusion proteins in gels, for identifying *E. coli* in agarose-containing medium [108] and for assaying lysosomal enzyme release from neutrophils.[91,92] The ELF substrates are patented by Molecular Probes.

Fluorogenic β-Glucosidase Substrates

β-Glucosidase, which is a marker for the endoplasmic reticulum (Section 12.4), is present in nearly all species. Its natural substrate is a glucosylceramide (Section 13.3). People with Gaucher's disease have mutations in the acid β-glucosidase gene that result in abnormal lysosomal storage.[9,109] Enzyme replacement therapy in Gaucher's disease patients [110] requires sensitive and selective methods for measuring β-glucosidase activity (Table 10.1). Plant β-glucosidases are implicated in a variety of key metabolic events and growth-related responses.[111]

Fluorescein Diglucoside

As with the other fluorescein diglycosides, Molecular Probes' fluorogenic fluorescein di-β-D-glucopyranoside (FDGlu, F-2881) is likely to yield the greatest sensitivity for detecting β-glucosidase activity in both cells [109] and cell extracts. This substrate has been used to demonstrate the utility of *Saccharomyces cerevisiae* and *Candida albicans* exo-1,3-β-glucanase genes as reporter genes.[112] Because these reporter genes encode secreted proteins, assays for reporter gene expression do not require cell permeabilization. FDGlu has been reported to be a selective substrate for the flow cytometric assay of lysosomal glucocerebrosidase activity in a variety of cells.[113] The assay demonstrated the inordinately low glucocerebrosidase activity present in fibroblasts of Gaucher's disease patients.

PFB Aminofluorescein Diglucoside

Through a collaboration with Matthew Lorincz and Leonard A. Herzenberg at Stanford University Medical School,[114] our patented PFB aminofluorescein diglucoside (PFB-FDGlu, P-11947) has proven to be an excellent substrate for the flow cytometric discrimination of normal peripheral blood mononuclear cells (PBMC) from the PBMC of patients with Gaucher's disease, a genetic deficiency in lysosomal β-glucocerebrosidase activity.[64] These researchers loaded the nonfluorescent PFB-FDGlu substrates into cells by pinocytosis, and then observed the green-fluorescent hydrolysis products in endosomal and lysosomal compartments. Under similar loading conditions, we have shown that the hydrolysis products of PFB aminofluorescein digalactoside (PFB-FDG, P-11948; Figure 10.18) and of PFB aminofluorescein diglucuronide (PFB-FDGlcU, P-11949) are similarly localized to endosomes and lysosomes.

Fluorescent Glucocerebrosides

The natural substrates for glucocerebrosidase are sphingosyl β-D-glucopyranosides. Our BODIPY FL analogs of this molecule — BODIPY FL C_5-glucocerebroside [115,116] (D-7548) and BODIPY FL C_{12}-glucocerebroside (D-7547) — are likely to be substrates for this lysosomal enzyme, which is lacking in Gaucher's disease patients. However, the lack of spectral shift of the hydrolysis products

— BODIPY FL C_5-ceramide (D-3521, Section 13.2) and BODIPY FL C_{12}-ceramide — means that extraction and chromatographic separation of the products is necessary for assessment of the activity.

Detection of Glucose and Glucose-Pr ducing Enzymes

Amplex Red Reagent for Glucose

The Amplex Red reagent (10-acetyl-3,7-dihydroxyphenoxazine, A-12222, A-22177; Section 10.5; Figure 10.49) is a colorless, stable and extremely versatile peroxidase substrate.[117] In an application similar to our use of the Amplex Red reagent to detect galactose-producing enzymes (see above), we have shown that it is practical to detect free glucose with high specificity at levels as low as 0.5 µg/mL using the Amplex Red reagent in combination with glucose oxidase (Figure 10.23). Because the peroxidase- and glucose oxidase–mediated reactions can be coupled, it is potentially possible to measure the release of glucose by any glucosidase enzyme — for instance, α-glucosidase, β-glucosidase and glucocerebrosidase — in either a continuous or discontinuous assay (Figure 10.50). This assay should also be very useful for quantitation of glucose levels in foods, fermentation media and bodily fluids. The long-wavelength spectral properties of resorufin (Figure 10.5) and high sensitivity of the assay result in little interference from colored components in the samples.

Amplex Red Glucose/Glucose Oxidase Assay Kit

Our Amplex Red Glucose/Glucose Oxidase Assay Kit (A-22189) provides all the reagents required for the assay of glucose and enzymes that produce glucose. The kit is also useful for the assay of glucose oxidase activity from cell extracts. We have even shown that the Amplex Red reagent can detect glucose liberated from native dextrans by dextranase [118] and from carboxymethylcellulose. The Amplex Red Glucose/Glucose Oxidase Assay Kit contains:

- The Amplex Red reagent
- DMSO and a concentrated reaction buffer
- D-glucose
- Glucose oxidase
- Horseradish peroxidase (HRP)
- H_2O_2 for use as a positive control
- A detailed protocol for the assays

The kit provides a sufficient amount of each reagent for approximately 500 assays using a reaction volume of 100 µL per assay and can be used with either an absorption- or fluorescence-based microplate reader or a fluorometer.

Amplex Red Neuraminidase/Sialidase Assay Kit

Neuraminidase (NA, also known as sialidase) is a very common enzyme that hydrolyzes terminal sialic acid residues on polysaccharide chains, most often exposing a galactose residue. Although NA is found in mammals, it is predominantly expressed in microorganisms such as bacteria and viruses.[119] Anti-influenza drug design has focused on the inhibition of both hemagglutinin and neuraminidase.[120] Various methods using chemiluminescence, absorption, and fluorescence have been developed to quantitate NA in biological fluids for detection of influenza virus [121,122] and for screening inhibitors of NA activity in drug development.[122,123] The ultimate goal has been to develop a rapid, single-step assay that is sensitive and adaptable for a high-throughput screening format. The development of an assay system utilizing the Amplex Red reagent with superior spectral and chemical characteristics meets these needs.

The Amplex Red Neuraminidase (Sialidase) Assay Kit (A-22178) provides an ultrasensitive method for detecting NA activity. This assay then utilizes the Amplex Red reagent to detect H_2O_2 generated by galactose oxidase–mediated oxidation of desialated galactose, the end result of NA action. In the presence of HRP, the H_2O_2 thus produced reacts with a 1:1 stoichiometry with the Amplex Red reagent to generate the red-fluorescent oxidation product, resorufin.[55] Resorufin has absorption and fluorescence emission maxima of approximately 563 nm and 587 nm, respectively (Figure 10.5), and because the extinction coefficient is high (54,000 $cm^{-1}M^{-1}$), the assay can be performed either fluorometrically or spectrophotometrically. In a purified system with fetuin as the substrate, NA levels as low as 0.2 mU/mL have been detected with the Amplex Red Neuraminidase (Sialidase) Assay Kit (Figure 10.24). NA activity can also be detected in biological samples such as serum (Figure 10.25). Kit contents include:

- The Amplex Red reagent
- Dimethylsulfoxide (DMSO)
- Horseradish peroxidase (HRP)
- Hydrogen peroxide (H_2O_2)
- A 5x reaction buffer
- Galactose oxidase from *Dactylium dendroides*
- Fetuin from fetal calf serum
- Neuraminidase from *Clostridium perfringens*
- A detailed protocol

Each kit provides sufficient reagents for approximately 400 assays using either a fluorescence or absorbance microplate reader and reaction volumes of 100 µL per assay.

Fluorogenic Chitinase/*N*-Acetylglucosaminidase Substrate

Chitin is the second most abundant organic compound in nature [124] and various chitinases and *N*-acetylglucosaminidases are widely distributed in bacteria, plants and eukaryotic cells. We have utilized our proprietary ELF technology (Section 6.3) to prepare the ELF 97 chitinase/*N*-acetylglucosaminidase substrate (ELF 97 *N*-acetylglucosaminide, ELF 97 NAG; E-22011), which is designed to allow spatially resolved detection of enzyme activity on colony indicator plates and histochemical analysis specimens. Other fluorogenic substrates for these enzymes [125,126] generate diffusible products and are therefore unsuitable for applications of this type. In addition to the capacity for localized precipitation at sites of enzymatic activity, the ELF 97 alcohol product that is generated upon hydrolysis of ELF 97 NAG is extremely photostable (Figure 6.18) and has widely separated fluorescence excitation and emission peaks (~360/520 nm, Figure 6.17). These properties make the signal easy to discriminate from any background fluorescence. ELF 97 NAG has been utilized to differentiate chitinase-active and non-chitinase–active subpopulations of a marine bacterium during chitin degradation.[127–129]

EnzChek Amylase Assay Kit

α-Amylase is a hydrolytic enzyme that catalyzes the conversion of starch to a mixture of glucose, maltose, maltotriose and dextrins. The levels of α-amylase in various fluids of the human body are of clinical importance,[130–132] while plant and microbial α-amylases are important enzymes for industry.[133]

Our EnzChek Amylase Assay Kit (E-11954) provides the speed, high sensitivity and convenience required for measuring α-amylase activity or for screening inhibitors in a high-throughput format. This EnzChek kit contains a starch derivative that is labeled with the BODIPY FL dye to such a degree that the fluorescence is quenched (Figure 10.47). α-Amylase–catalyzed hydrolysis relieves this quenching, yielding brightly fluorescent BODIPY FL dye–labeled fragments. The accompanying increase in fluorescence is proportional to amylase activity and can be monitored with a

fluorescence microplate reader, minifluorometer or standard fluorometer. Each EnzChek Amylase Assay Kit includes:

- A heavily labeled BODIPY FL conjugate of starch from corn (DQ starch)
- A 10× reaction buffer
- α-Amylase from *Bacillus* sp., for use as a positive control
- A detailed protocol

A sufficient amount of each reagent is supplied for ~1000 assays using 200 µL assay volumes and 96-well microplates or ~100 assays using 2 mL assay volumes and standard fluorescence cuvettes.

Using 12.5 µg/mL of the DQ starch substrate and a 60-minute incubation period at room temperature, the assay can detect the activity of this enzyme down to a final concentration of 1×10^{-4} U/mL (~0.3 µg protein/mL), where one unit is defined as the amount of enzyme required to liberate 1 mg of maltose from starch in 3 minutes at 20°C, at pH 6.9.

EnzChek Lysozyme Assay Kit

Lysozyme (muramidase) is an important but difficult enzyme to assay. Lysozyme hydrolyzes β-1-4-glycosidic linkages between *N*-acetylmuramic acid and *N*-acetyl-D-glucosamine residues present in the mucopolysaccharide cell wall of a variety of microorganisms. Lysozyme is present in human serum, urine, tears, seminal fluid and milk. Serum and urine lysozyme levels may be elevated in acute myelomonocytic leukemia (FAB-M4), chronic myelomonocytic leukemia (CMML) and chronic myelocytic leukemia (CML).[134] Increased serum lysozyme activity is also present in tuberculosis,[135] sarcoidosis, megaloblastic anemias,[136] acute bacterial infections, ulcerative colitis [137] and Crohn's disease.[135,137] Elevated levels of urine and serum lysozyme occur during severe renal insufficiency, renal transplant rejection,[138] urinary tract infections,[139] glomerulonephritis and nephrosis.[140]

Molecular Probes has developed a simple and sensitive assay that can continuously measure the activity of lysozyme in solution. Our fluorescence-based EnzChek Lysozyme Assay Kit (E-22013) permits the detection of as little as 30 U/mL of lysozyme (Figure 10.26). One unit of lysozyme is the quantity of enzyme that produces a decrease in turbidity of 0.001 optical density units per minute at 450 nm measured at pH 7.0 (25°C) using a 0.3 mg/mL suspension of *Micrococcus lysodeikticus* cells as substrate.[141] This assay measures lysozyme activity on *M. lysodeikticus* cell walls that are labeled to such a degree that fluorescence is quenched. Lysozyme action can relieve the quenching, yielding a dramatic increase in fluorescence that is proportional to lysozyme activity. This increase in fluorescence can be measured with any spectrofluorometer, mini-fluorometer or fluorescence microplate reader that can detect fluorescein (excitation/emission maxima ~490/525 nm).

The EnzChek Lysozyme Assay Kit (E-22013) contains:

- The DQ lysozyme substrate — a fluorescein conjugate of *Micrococcus lysodeikticus* so heavily labeled that its fluorescence is quenched
- Reaction buffer
- Lysozyme from chicken egg white
- A detailed protocol for the assay

Each kit contains sufficient materials for approximately 400 assays of 100 µL in a fluorescence microplate reader.

Chrom genic Glyc sidase Substrates

The widely used β-galactosidase substrate — 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside

(X-Gal, B-1690, B-22015) — yields a dark blue precipitate at the site of enzymatic activity. X-Gal is useful for numerous histochemical and molecular biology applications, including detection of *lacZ* activity in cells and tissues. In contrast to β-glucuronidase as a gene marker, β-galactosidase can be fixed in cells and tissues with glutaraldehyde without loss of activity and detected with high resolution with X-Gal.[142] Molecular Probes offers X-Gal at an attractive price, particularly in our 25-gram bulk packaging size (B-22015).

The chromogenic substrate 5-bromo-4-chloro-3-indolyl β-D-glucuronic acid (X-GlcU, B-1691) forms a dark blue precipitate. X-GlcU is routinely used to detect GUS expression in transformed plant cells and tissues.[143–149] However, because it is relatively difficult to differentiate the blue color of the product of X-GlcU against the dark green chloroplasts,[150] we also offer the isomeric 5-bromo-6-chloro-3-indolyl β-D-glucuronide [151] (B-8408), which forms a magenta-colored precipitate. X-GlcU can also be used to detect *E. coli* contamination in food and water.[108,152]

Auxiliary Products for Glycosidase Research

Phenylethyl β-D-Thiogalactopyranoside (PETG)

Phenylethyl β-D-thiogalactopyranoside (PETG, P-1692) is a cell-permeant inhibitor of β-galactosidase activity.[33,153] We provide PETG in our FluoReporter, DetectaGene and ImaGene *lacZ* Gene Expression Kits for stopping the enzymatic reaction.

β-Galactosidase and Its Streptavidin Conjugate

Molecular Probes also offers the streptavidin conjugate of β-galactosidase (S-931), a reagent used in a variety of ELISAs.[154] Streptavidin–β-D-galactosidase reportedly provided enhanced sensitivity over that obtained with the avidin conjugate of HRP in the detection of a variety of mammalian interleukins and their receptors by ELISA.[155] This reagent has also been used in fluorometric-reverse (IgE-capture) [156] and fluorescence-sandwich [157] ELISAs.

Rabbit Anti–β-Galactosidase Antibody

Molecular Probes offers a polyclonal antibody to the widely used reporter gene product, β-galactosidase. Our rabbit anti–β-galactosidase antibody (A-11132) is raised against *E. coli*–derived β-galactosidase and demonstrates high selectivity for the enzyme. Whether it is being used as a reporter gene or to generate fusion proteins, anti–β-galactosidase provides an easy tool for detecting the enzyme. The antibody is suited to a variety of techniques, including immunoblotting, ELISA, immunoprecipitation and most immunological methods. β-Galactosidase has been used as a tag for quantitative detection of molecules expressed on a cell surface in unfixed, live cells, using anti–β-galactosidase and a β-galactosidase substrate for detection.[158] This novel "cell-ELISA" technique is reported to be applicable to adherent cells and nonadherent cells and to have utility for large-scale screening for expression of cell-surface molecules and of hybridomas for production of antibodies to cell-surface epitopes.

Rabbit Anti–β-Glucuronidase Antibody

In combination with a fluorophore- or enzyme-labeled anti–rabbit IgG secondary antibody (Section 7.3, Table 7.6), our anti–β-glucuronidase antibody (A-5790) can be used to detect the GUS enzyme in transformed plant tissue [159,160] and in transfected animal cells [161] using Western blotting or immunohistochemical techniques. Furthermore, this antibody, which is raised in rabbits against *E. coli*–type X-A β-glucuronidase, can be immobilized in microplate wells in order to capture the GUS enzyme from cell lysates.[162] The enzymatic activity can subsequently be determined using any of our fluorogenic or chromogenic β-glucuronidase substrates.[163]

ManLev: A Metab lically Active Carb hydrate Anal g

N-Levulinoyl-D-mannosamine [164] (ManLev, L-20492; Section 3.2, Figure 3.9) and *N*-levulinoyl-D-mannosamine, tetraacetate (ManLev tetraacetate, L-20493; Section 3.2) are ketone-containing monosaccharides that serve as substrates in the oligosaccharide synthesis pathway, resulting in ketone-tagged cell-surface oligosaccharides.[165–168] Since ketones are rare in cells, reaction with 2 µM biotinylated aldehyde-reactive probe (ARP, A-10550; Section 3.2; Figure 3.10) followed by a fluorescent avidin or streptavidin conjugate (Section 7.6) provides a means of identifying and tracing tagged cells by either imaging (Figure 3.11) or flow cytometry.

Related Products for Carbohydrate Research

Molecular Probes offers an extensive assortment of reagents for detection and analysis of carbohydrates that are described in other sections of this *Handbook*. These products include:

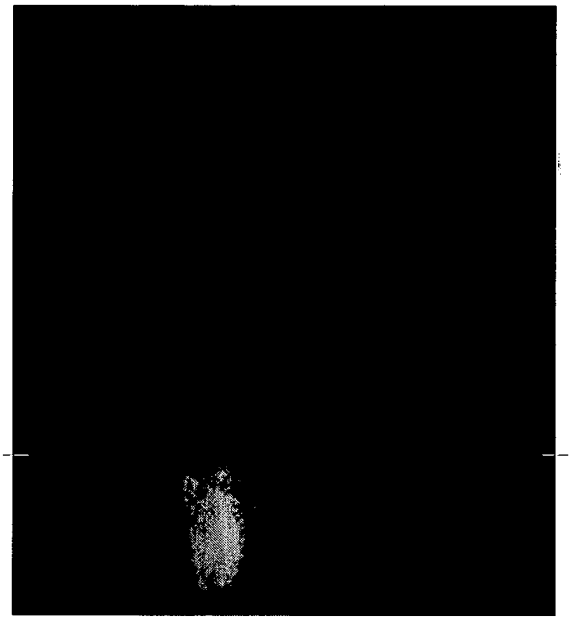
- Hydrazine and aromatic amine reagents for derivatization and analysis of carbohydrates by electrophoretic methods (Section 3.2, Table 3.1)
- Lectins and fluorescent lectin conjugates (Section 7.7, Table 7.21)
- Pro-Q Glycoprotein Blot and Gel Stain Kits (Section 9.4)
- Pro-Q Emerald 300 Lipopolysaccharide Gel Stain Kit (P-20495, Section 3.2)
- Fluorescent lipopolysaccharides (Section 13.3, Section 16.1; Table 16.1)
- Fluorescent glycolipids, including phosphatidyl inositol derivatives (Section 13.2, Section 13.3)
- BODIPY FL C_5-ganglioside G_{M1} (B-13950, Section 13.3)
- Fluorescent and biotinylated dextrans (Section 14.5, Table 14.4)
- NBD-glucosamine derivatives for glucose-transport studies (N-13195, N-23106; Section 15.2)
- Fluorescein insulin (I-13269, Section 16.1)
- Fluorescent phosphatidylinositol phosphate derivatives (Table 13.2) and antibodies to phosphatidylinositol phosphates (Section 18.4)

1. J Clin Microbiol 30, 1402 (1992); **2.** Microbiol Rev 55, 335 (1991); **3.** Meth Microbiol 19, 105 (1987); **4.** Nature 369, 113 (1994); **5.** J Cell Biol 110, 309 (1990); **6.** Anal Biochem 148, 50 (1985); **7.** Traffic 1, 836 (2000); **8.** Clin Chim Acta 205, 87 (1992); **9.** Crit Rev Biochem Mol Biol 25, 385 (1990); **10.** Mol Gen Genet 216, 321 (1989); **11.** Mol Gen Genet 215, 38 (1988); **12.** Plant Mol Biol 10, 387 (1988); **13.** Proc Natl Acad Sci U S A 83, 8447 (1986); **14.** J Immunol Methods 150, 5 (1992); **15.** Anal Biochem 257, 234 (1998); **16.** J Immunol Methods 150, 23 (1992); **17.** J Immunol Methods 48, 133 (1982); **18.** Exp Parasitol 73, 440 (1991); **19.** Dev Biol 161, 77 (1994); **20.** Mol Microbiol 13, 655 (1994); **21.** J Neurosci 13, 1418 (1993); **22.** Eur J Immunol 19, 1619 (1989); **23.** Cytometry 17, 216 (1994); **24.** Eur J Cell Biol 62, 324 (1993); **25.** J Biol Chem 268, 9762 (1993); **26.** Neuron 9, 1117 (1992); **27.** Science 251, 81 (1991); **28.** Proc Natl Acad Sci U S A 85, 2603 (1988); **29.** J Virol Methods 44, 99 (1993); **30.** Proc Natl Acad Sci U S A 90, 8194 (1993); **31.** Appl Environ Microbiol 56, 3861 (1990); **32.** Purchase of the FluoReporter *lacZ* Flow Cytometry Kits is accompanied by a research license under US 5,070,012; **33.** Carbohydr Res 56, 153 (1977); **34.** Methods 2, 261 (1991); **35.** Methods 2, 248 (1991); **36.** Anal Chim Acta 163, 67 (1984); **37.** Oncogene 10, 2323 (1995); **38.** Cytometry 9, 394 (1988); **39.** Ann N Y Acad Sci 613, 333 (1990); **40.** Anal Chim Acta 213, 245 (1988); **41.** DDAO-based substrates are licensed to Molecular Probes under US 4,810,636 and its foreign equivalents; **42.** Anal Biochem 215, 24 (1993); **43.** Neuron 10, 427 (1993); **44.** Proc Natl Acad Sci U S A 84, 156 (1987); **45.** J Histochem Cytochem 33, 965 (1985); **46.** Clin Chim Acta 12, 647 (1965); **47.** Bioorg Med Chem Lett 8, 3107 (1998); **48.** Anal Lett 21, 193 (1988); **49.** J Histochem Cytochem 34, 585 (1986); **50.** Biotechniques 30, 776 (2001); **51.** Infect Immun 61, 5231 (1993); **52.** Anal Biochem 146, 211 (1985); **53.** Proc Natl Acad Sci U S A 95, 6373 (1998); **54.** J Biochem (Tokyo) 100, 707 (1986); **55.** J Immunol Methods 202, 133 (1997); **56.** J Fluorescence 3, 119 (1993); **57.** Development 119, 1217 (1993); **58.** Neuron 14, 685 (1995); **59.** J Neurosci 15, 1025 (1995); **60.** Exp Cell Res 219, 372 (1995); **61.** Science 266, 1881 (1994); **62.** Cytometry 28, 36 (1997); **63.** J Histochem Cytochem 44, 1363 (1996); **64.** US 5,773,236; **65.** Lysosomes, Holtzman E pp. 319–361 (1989); **66.** FASEB J 5, 3108 (1991); **67.** Appl Environ Microbiol 60, 4638 (1994); **68.**

Biotechnol Bioeng 42, 1113 (1993); **69.** J Clin Endocrinol Metab 78, 1232 (1994); **70.** Biotechnol Bioeng 42, 708 (1993); **71.** J Biol Chem 271, 29312 (1996); **72.** Proc Natl Acad Sci U S A 89, 10681 (1992); **73.** Methods Cell Biol 42 Pt B, 563 (1994); **74.** Biotechniques 14, 274 (1993); **75.** Genes Dev 6, 591 (1992); **76.** J Lab Clin Med 110, 740 (1987); **77.** Cytometry 20, 324 (1995); **78.** Hum Mol Genet 5, 875 (1996); **79.** FEMS Microbiol Lett 179, 317 (1999); **80.** US 5,208,148 and US 5,242,805; **81.** Cytometry 12, 184 (1991); **82.** Cytometry 15, 349 (1994); **83.** Biotechniques 8, 37 (1990); **84.** Plant Mol Biol Rep 5, 387 (1988); **85.** Tetrahedron 53, 7159 (1997); **86.** Electrophoresis 22, 970 (2001); **87.** Electrophoresis 21, 497 (2000); **88.** J Biochem Biophys Methods 33, 197 (1996); **89.** Anal Biochem 278, 123 (2000); **90.** Biotechniques 30, 474 (2001); **91.** Cell Signal 3, 625 (1991); **92.** J Immunol Methods 100, 211 (1987); **93.** Cytometry 24, 321 (1996); **94.** J Biol Chem 274, 657 (1999); **95.** J Assoc Off Anal Chem 71, 589 (1988); **96.** Appl Environ Microbiol 50, 1383 (1985); **97.** Can J Microbiol 39, 1066 (1993); **98.** J Microbiol Methods 12, 51 (1990); **99.** J Microbiol Methods 12, 235 (1990); **100.** Biotechniques 16, 888 (1994); **101.** GUS Protocols: Using the GUS Gene as a Reporter of Gene Expression, Gallapher SR, Ed. pp. 61–76 (1992); **102.** Plant Sci 78, 73 (1991); **103.** Plant Mol Biol 15, 527 (1990); **104.** Plant Mol Biol Rep 10, 37 (1992); **105.** Plant J 10, 745 (1996); **106.** Microbiology 143, 267 (1997); **107.** Ann N Y Acad Sci 346, 419 (1980); **108.** J Appl Bacteriol 74, 223 (1993); **109.** Cell Biochem Funct 11, 167 (1993); **110.** Neurochem Res 24, 301 (1999); **111.** 533 (1993); **112.** Yeast 10, 747 (1994); **113.** Anal Biochem 247, 268 (1997); **114.** Blood 89, 3412 (1997); **115.** Frontiers in Bioactive Lipids, Vanderhoek JV, Ed. pp. 203–213 (1996); **116.** J Cell Biol 125, 769 (1994); **117.** Anal Biochem 253, 162 (1997); **118.** Anal Biochem 260, 257 (1998); **119.** J Biochem Biophys Methods 22, 23 (1991); **120.** Nat Biotechnol 18, 835 (2000); **121.** Antiviral Res 47, 1 (2000); **122.** J Med Chem 13, 697 (1970); **123.** Anal Biochem 280, 291 (2000); **124.** Trends Biotechnol 16, 301 (1998); **125.** Appl Environ Microbiol 64, 613 (1998); **126.** Anal Biochem 208, 74 (1993); **127.** Methods Enzymol 336, 279 (2001); **128.** Appl Environ Microbiol 66, 3566 (2000); **129.** Appl Environ Microbiol 66, 3574 (2000); **130.** Medicine (Baltimore) 55, 269 (1976); **131.** Clin Chem 21, 57 (1976); **132.** Clin Chem 30, 387 (1984); **133.** Starch/Starke 35, 169 (1983); **134.** Mod Pathol 7, 771 (1994); **135.** Acta Pathol Jpn 28, 689 (1978); **136.** N Engl J Med 277, 10 (1967); **137.** J Clin Pathol 36, 1312 (1983); **138.** Clin Chem 32, 1807 (1986); **139.** Toxicology 28, 347 (1983); **140.** Nephron 63, 423 (1993); **141.** Biochim Biophys Acta 8, 302 (1952); **142.** EMBO J 8, 343 (1989); **143.** Biotechniques 19, 106 (1995); **144.** Biotechnology (N Y) 8, 833 (1990); **145.** Plant Cell Physiol 31, 805 (1990); **146.** Science 249, 1285 (1990); **147.** Science 248, 471 (1990); **148.** Nature 342, 837 (1989); **149.** EMBO J 6, 3901 (1987); **150.** Biotechniques 7, 922 (1989); **151.** Biotechniques 19, 352 (1995); **152.** Lett Appl Microbiol 13, 212 (1991); **153.** Anal Biochem 199, 119 (1991); **154.** J Immunol Methods 125, 279 (1989); **155.** Biochemistry 26, 4922 (1987); **156.** J Immunol Methods 116, 181 (1989); **157.** J Immunol Methods 110, 129 (1988); **158.** J Immunol Methods 234, P153 (2000); **159.** J Biol Chem 269, 17635 (1994); **160.** Plant Mol Biol 15, 821 (1990); **161.** Biotechniques 27, 896 (1999); **162.** J Clin Microbiol 32, 1444 (1994); **163.** Appl Environ Microbiol 53, 1073 (1987); **164.** US 6,075,134; **165.** Glycobiology 11, 11R (2001); **166.** Nat Biotechnol 19, 553 (2001); **167.** Methods Enzymol 327, 260 (2000); **168.** Glycobiology 10, 1049 (2000).

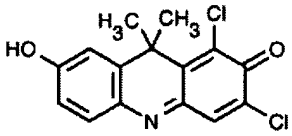
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Figure 10.10 Sternomastoid muscle fibers of a living mouse that have been transfected with YOYO-1 dye–stained DNA (red) containing the *lacZ* reporter gene and then stained with the β-galactosidase substrate fluorescein di-β-D-galactopyranoside (FDG, F-1179). DNA stained with YOYO-1 (Y-3601) prior to implantation could still be localized five days after application. Fluorescence signals were visualized *in situ* by epifluorescence microscopy with a low–light level SIT camera and a computer imaging system. Image contributed by Peter van Mier, Washington University School of Medicine.



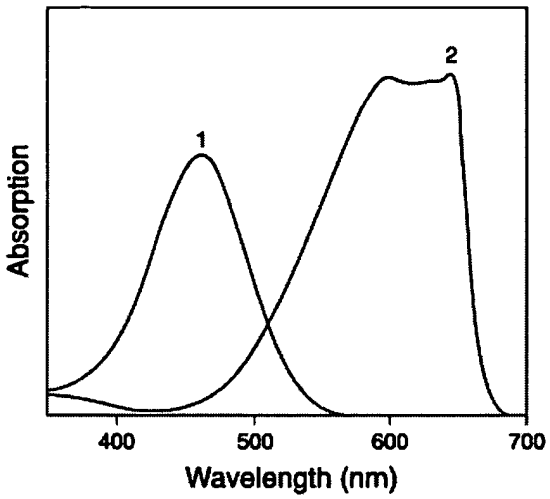
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Figure 10.11 H-6482 7-hydroxy-9*H*-(1,3-dichloro-9,9-dimethylacridin-2-one).



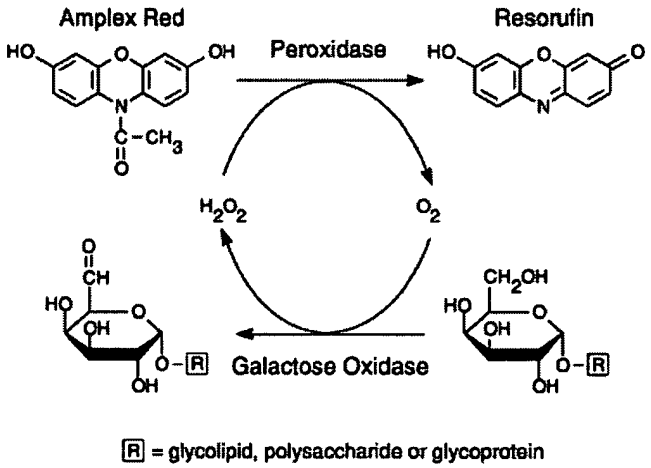
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Figure 10.12 Absorption spectra of **1)** DDAO galactoside (D-6488) and **2)** DDAO (H-6482) at equal concentrations in pH 9 aqueous buffer. These spectra show the large spectral shift accompanying enzymatic cleavage of DDAO-based substrates. DDAO phosphate (D-6487) has very similar spectra.



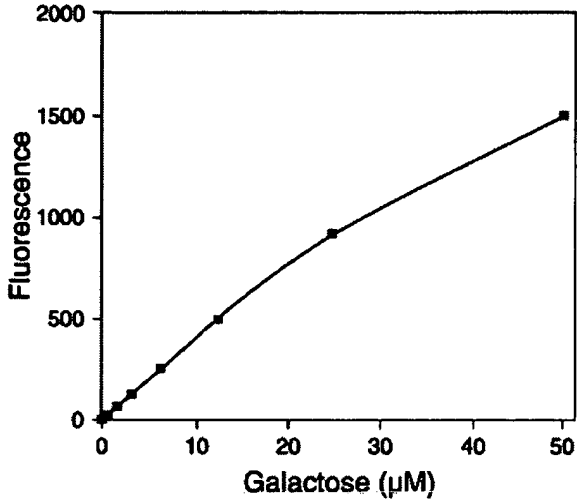
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Figure 10.13 Detection scheme utilized in the Amplex Red Galactose/Galactose Oxidase Assay Kit (A-22179). Oxidation of the terminal galactose residue of a glycoprotein, glycolipid or polysaccharide results in the generation of H_2O_2, which, in the presence of horseradish peroxidase (HRP), reacts stoichiometrically with the Amplex Red reagent to generate the red-fluorescent oxidation product, resorufin.



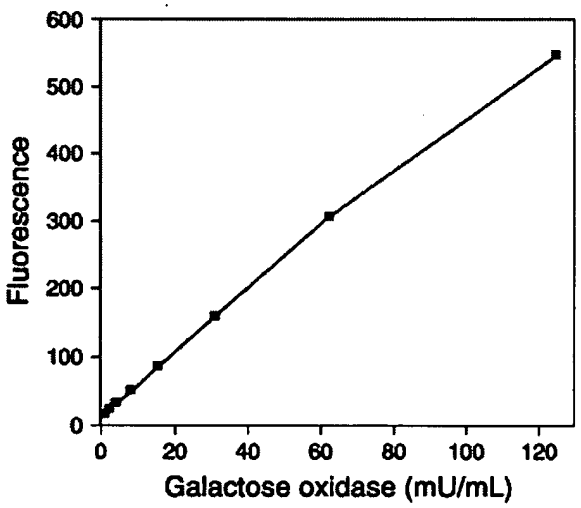
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Figure 10.14 Detection of galactose using the Amplex Red Galactose/Galactose Oxidase Assay Kit (A-22179). Each reaction contained 50 µM Amplex Red reagent, 0.1 U/mL HRP, 2 U/mL of galactose oxidase and the indicated amount of galactose in 1X Reaction Buffer. Reactions were incubated at 37°C. After 30 minutes, fluorescence was measured in a fluorescence microplate reader using excitation at 530 ± 12.5 nm and fluorescence detection at 590 ± 17.5 nm. A background fluorescence of 93 units was subtracted from each data point.



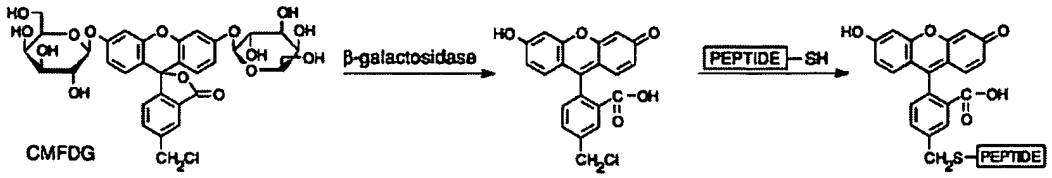
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Figure 10.15 Detection of galactose oxidase activity using the Amplex Red Galactose/Galactose Oxidase Assay Kit (A-22179). Each reaction contained 50 µM Amplex Red reagent, 0.1 U/mL HRP, 100 µM galactose and the indicated amount of galactose oxidase in 1X Reaction Buffer. Reactions were incubated at 37°C. After 20 minutes, fluorescence was measured in a fluorescence microplate reader using excitation at 530 ± 12.5 nm with fluorescence detection at 590 ± 17.5 nm.



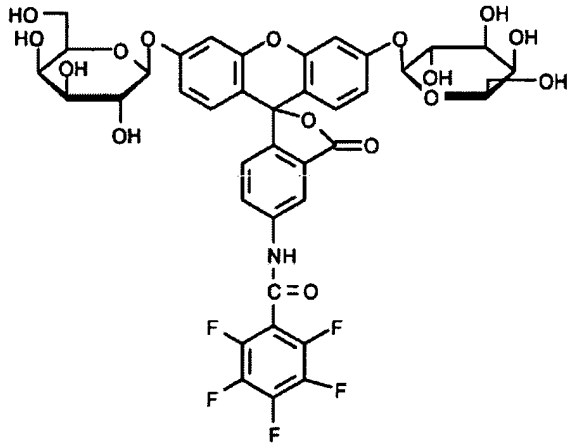
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Figure 10.16 Sequential β-galactosidase hydrolysis and peptide conjugate formation of CMFDG (D-2920).



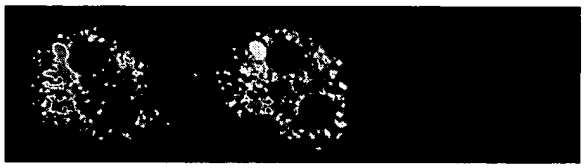
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Figure 10.17 P-11948 5-(pentafluorobenzoylamino)fluorescein di-β-D-galactopyranoside.



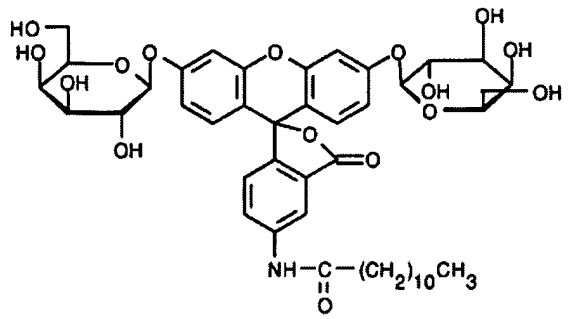
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Figure 10.18 Bovine pulmonary artery endothelial cells simultaneously stained with LysoTracker Red DND-99 (L-7528), a cell-permeant, fixable lysosomal stain, and with 5-(pentafluorobenzoylamino) fluorescein di-β-D-galactopyranoside (PFB-FDG, P-11948), a fluorogenic substrate for β-galactosidase. PFB-FDG is nonfluorescent until enzymatically hydrolyzed to green-fluorescent PFBF. The center image demonstrates colocalization of the LysoTracker Red DND-99 dye and the fluorescent product, PFB-F, to the lysosomes. The left image was acquired with a bandpass filter set appropriate for fluorescein, the right image was acquired with a bandpass filter set appropriate for Texas Red dye, and the center image was acquired with a triple bandpass optical filter set appropriate for DAPI, fluorescein and the Texas Red dye.



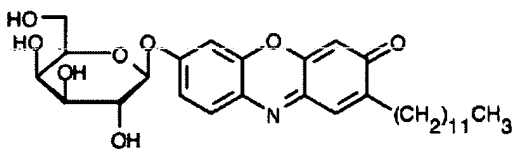
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Figure 10.19 I-2904 ImaGene Green C_{12}FDG *lacZ* Gene Expression Kit.



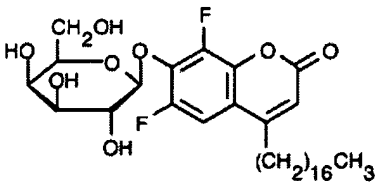
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Figure 10.20 I-2906 ImaGene Red C_{12}RG *lacZ* Gene Expression Kit.



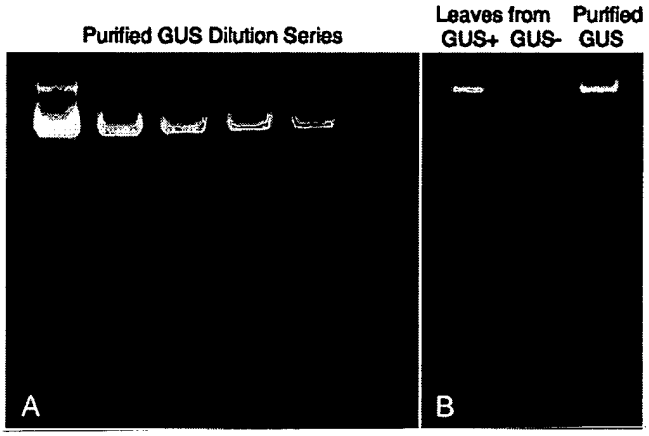
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Figure 10.21 D-11950 6,8-difluoro-4-heptadecylumbelliferyl β-D-galactopyranoside.



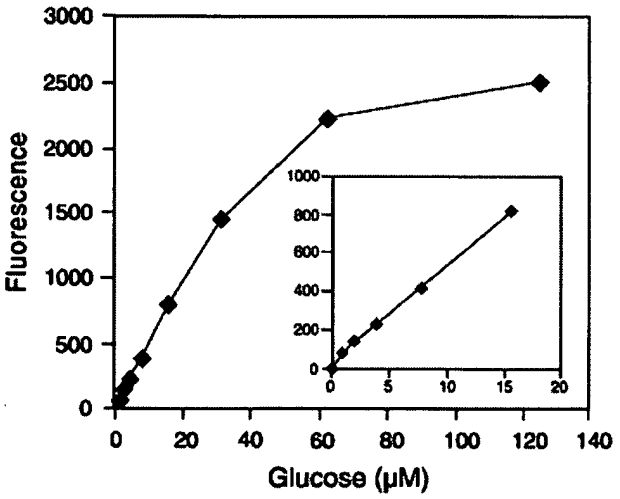
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Figure 10.22 *In situ* gel assay of β-D-glucuronidase (GUS) activity with ELF 97 β-D-glucuronide (E-6587). **A)** Twofold dilutions of the purified GUS enzyme or **B)** cell extracts from single leaves from GUS-positive and -negative *Arabidopsis* plants were electrophoresed through a native 7.5% polyacrylamide gel. Following electrophoresis, the gel was washed with 0.1 M sodium phosphate, pH 7.0, containing 0.2% Triton X-100, at room temperature for 60 minutes and then incubated with 15 µM ELF 97 β-D-glucuronide in 0.1 M sodium phosphate, pH 7.0, at 37°C, for 30–60 minutes. The gel was photographed using 300 nm transillumination, a SYBR photographic filter (S-7569, Section 8.4) and Polaroid 667 black-and-white print film.



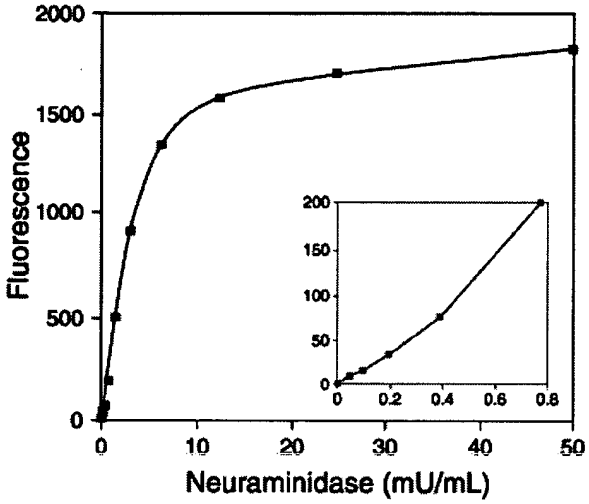
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Figure 10.23 Detection of glucose using the Amplex Red Glucose/Glucose Oxidase Assay Kit (A-22189). Reactions containing 50 µM Amplex Red reagent, 0.1 U/mL HRP, 1 U/mL glucose oxidase and the indicated amount of glucose in 50 mM sodium phosphate buffer, pH 7.4, were incubated for one hour at room temperature. Fluorescence was then measured with a fluorescence-based microplate reader using excitation at 530 ± 12.5 nm and fluorescence detection at 590 ± 17.5 nm. Background fluorescence (arbitrary units), determined for a no-glucose control reaction, has been subtracted from each value. The inset shows the sensitivity and linearity of the assay at low levels of glucose (0–15 µM).



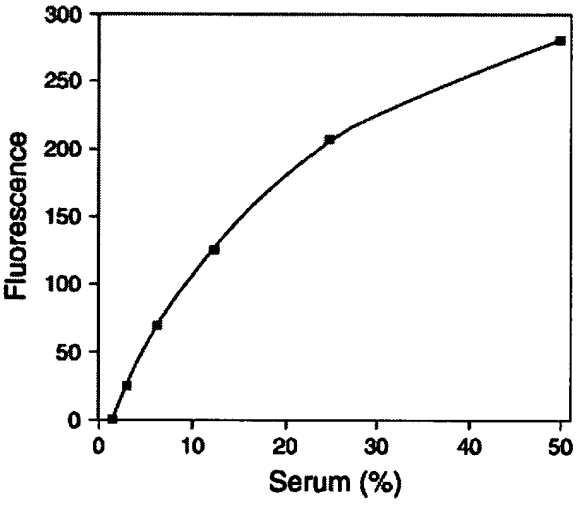
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Figure 10.24 Detection of neuraminidase activity using the Amplex Red Neuraminidase (Sialidase) Assay Kit (A-22178). Each reaction contained 50 µM Amplex Red reagent, 0.1 U/mL HRP, 2 U/mL galactose oxidase, 250 µg/mL fetuin and the indicated amount of neuraminidase in 1x Reaction Buffer. Reactions were incubated at 37°C. After 30 minutes, fluorescence was measured in a fluorescence-based microplate reader using excitation at 530 ± 12.5 nm and fluorescence detection at 590 ± 17.5 nm. A background fluorescence of 70 fluorescence units was subtracted from each data point.



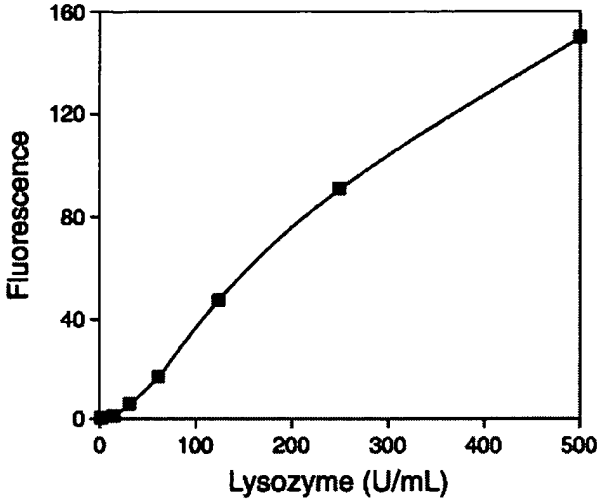
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Figure 10.25 Detection of neuraminidase activity in serum using the Amplex Red Neuraminidase (Sialidase) Assay Kit (A-22178). Each reaction contained 50 µM Amplex Red reagent, 0.1 U/mL HRP, 2 U/mL galactose oxidase, 250 µg/mL fetuin and the indicated amount of serum in 1x Reaction Buffer. Reactions were incubated at 37°C. After 60 minutes, fluorescence was measured in a fluorescence-based microplate reader using excitation at 530 ± 12.5 nm and fluorescence detection at 590 ± 17.5 nm. A background fluorescence of 112 units was subtracted from each data point.



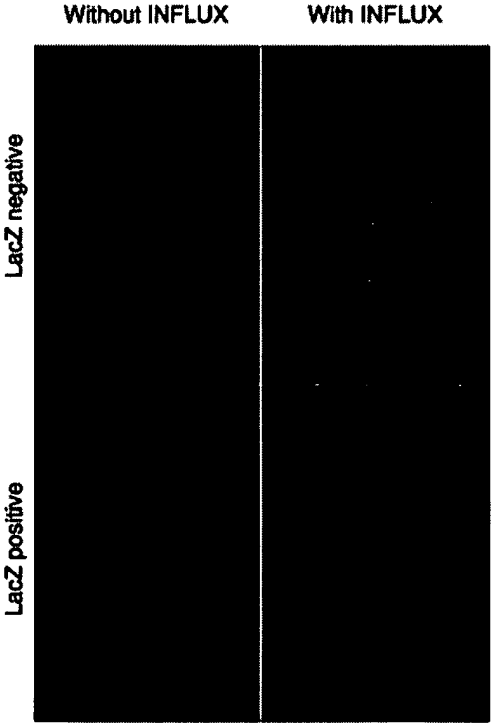
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Figure 10.26 Detection of lysozyme activity using the EnzChek Lysozyme Assay Kit (E-22013). Increasing amounts of lysozyme were incubated with the DQ lysozyme substrate for 60 minutes at 37°C. The fluorescence was measured in a fluorescence microplate reader using excitation/emission wavelengths of ~485/530 nm. Background fluorescence, determined for a no-enzyme control, was subtracted from each value.



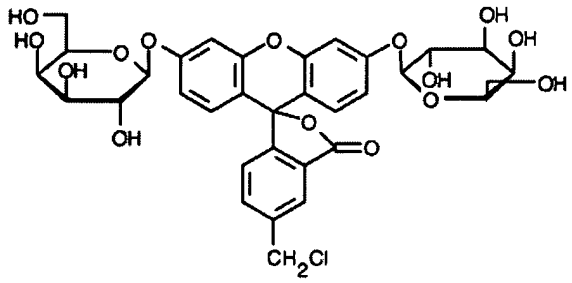
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Figure. *lacZ* negative (NIH 3T3) and *lacZ* positive (CRE BAG 2) cells loaded with 1 µg/mL CMFDG by diffusion or by the Influx pinocytic cell-loading reagent (I-14402), which is also a component of the DetectaGene Green CMFDG *lacZ* Gene Expression Kit (D-2920). The Influx reagent allows for better cytoplasmic distribution of CMFDG, resulting in a uniform signal in *lacZ*-positive cells. Images were acquired using a bandpass filter appropriate for fluorescein.



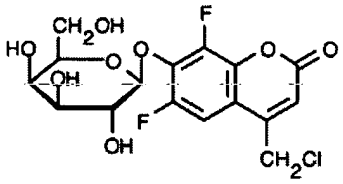
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Figure. D-2920 DetectaGene Green CMFDG *lacZ* Gene Expression Kit.



* * * * *

Figure. C-11946 4-chloromethyl-6,8-difluoroumbelliferyl β-D-galactopyranoside.



* * * * *

Table 10.1 — Glycosidase enzymes and their fluorogenic and chromogenic substrates

Carbohydrate (Enzyme)	Notes on Enzyme Activity	Labeled Substrate (Abs/	
β-D-Galactopyranoside (β-Galactosidase, E.C. 3.2.1.23)	• Useful as a reporter gene marker [1–4] • Useful for ELISAs [5–8] • Useful for enumerating coliforms from the family *Enterobacteriaceae* [9–11] • Useful for classifying mycobacteria [12]	Blue-fluorescent products	3-Carboxy
			4-Chlorom (371/464)
			6,8-Difluo (358/452)
			6,8-Difluo (366/454)
			4-Methylu
		Green-fluorescent products	Fluorescei
			5-(Pentafl (490/514)
			C_8-Fluores

			C_{12}-Fluore
			5-Chlorom
		Red-fluorescent products	C_{12}-Resoru
			DDAO (64
			Resorufin
		Chromogenic substrates	5-Bromo-4
		β-D-Glucopyranoside (β-Glucosidase, E.C. 3.2.1.31)	• Defici which storag disea • Usefu endop
5-(Pentafluorobenzoylamino)fluorescein (490/514)	P-11947 [29]		
β-D-Glucuronide (β-Glucuronidase, GUS; E.C. 3.2.1.31)	• Useful as a reporter gene marker [30] • Useful as a lysosomal marker [31,32] • Useful for detecting *E. coli*; 94–96% of *E. coli* contain this enzyme, but it is less common in *Shigella* (44–58%), *Salmonella* (20–29%) and *Yersinia* strains [9,33–35]	Blue-fluorescent products	6,8-Difluo (358/452)
			4-Methylu
			4-Trifluoro
		Green-fluorescent products	Fluorescei
			5-(Pentafl (490/514)
			C_{12}-Fluore
			ELF 97 (34
		Chromogenic substrates	5-Bromo-4
			5-Bromo-6

* Approximate absorption (Abs) and fluorescence emission (Em) maxima of enzymatic hydrolysis p Kit. ‡ ImaGene Gene Expression Kit. NA = Not applicable.

1. Cytometry 17, 216 (1994); **2.** Dev Biol 161, 77 (1994); **3.** Proc Natl Acad Sci U S A 85, 2603 (1988); **4.** Biophys J 74, 11 (1998); **5.** Anal Biochem 146, 211 (1985); **6.** Exp Parasitol 73, 440 (1991); **7.** J Immunol Methods 54, 297 (1982); **8.** J Virol Methods 3, 155 (1981); **9.** Microbiol Rev 55, 335 (1991); **10.** J Appl Bacteriol 64, 65 (1988); **11.** Appl Environ Microbiol 35, 136 (1978); **12.** Zentralbl Bakteriol 280, 476 (1994); **13.** Infect Immun 61, 5231 (1993); **14.** Anal Biochem 273, 41 (1999); **15.** Anal Biochem 104, 182 (1980); **16.** Anal Biochem 215, 24 (1993); **17.** Cytometry 20, 324 (1995); **18.** Appl Environ Microbiol 60, 4638 (1994); **19.** Proc Natl Acad Sci U S

A 89, 10681 (1992); **20.** FASEB J 5, 3108 (1991); **21.** J Neurosci 15, 1025 (1995); **22.** Biotechnol Bioeng 42, 1113 (1993); **23.** US Patent No. 5,242,805 (1993); **24.** Anal Chim Acta 163, 67 (1984); **25.** Biotechniques 7, 576 (1989); **26.** Cell Biochem Funct 11, 167 (1993); **27.** Nature 369, 113 (1994); **28.** Anal Biochem 247, 268 (1997); **29.** Blood 89, 3412 (1997); **30.** Plant Mol Biol Rep 5, 387 (1988); **31.** Cell Signal 3, 625 (1991); **32.** J Immunol Methods 100, 211 (1987); **33.** Appl Environ Microbiol 59, 3534 (1993); **34.** Appl Environ Microbiol 50, 1383 (1985); **35.** J Clin Microbiol 13, 483 (1981); **36.** J Appl Bacteriol 74, 223 (1993); **37.** Arch Biochem Biophys 286, 394 (1991); **38.** Plant Sci 78, 73 (1991); **39.** Biochem Int 24, 1135 (1991); **40.** J Biol Chem 274, 657 (1999); **41.** Microbiology 143, 267 (1997); **42.** Plant J 10, 745 (1996); **43.** Electrophoresis 21, 497 (2000); **44.** Tetrahedron 53, 7159 (1997); **45.** Biotechniques 19, 352 (1995).

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Product List — 10.2 Detecting Glycosidases

Cat #	Product Name	Unit Size
A-22010	5-acetylaminofluorescein di-β-D-galactopyranoside (C_2FDG)	5 mg
A-22179	Amplex® Red Galactose/Galactose Oxidase Assay Kit *400 assays*	1 kit
A-22189	Amplex® Red Glucose/Glucose Oxidase Assay Kit *500 assays*	1 kit
A-22178	Amplex® Red Neuraminidase (Sialidase) Assay Kit *400 assays*	1 kit
A-12222	Amplex® Red reagent (10-acetyl-3,7-dihydroxyphenoxazine)	5 mg
A-11132	anti-β-galactosidase, rabbit IgG fraction *2 mg/mL*	0.5 mL
A-5790	anti-β-glucuronidase, rabbit IgG fraction *2 mg/mL*	0.5 mL
B-1690	5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-Gal)	1 g
B-22015	5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-Gal) *bulk packaging*	25 g
B-1691	5-bromo-4-chloro-3-indolyl β-D-glucuronide, cyclohexylammonium salt (X-GlcU, CHA)	100 mg
B-8408	5-bromo-6-chloro-3-indolyl β-D-glucuronide, cyclohexylammonium salt	25 mg
C-1488	3-carboxyumbelliferyl β-D-galactopyranoside (CUG)	10 mg
C-11946	4-chloromethyl-6,8-difluoroumbelliferyl β-D-galactopyranoside (CMDiFUG)	5 mg
D-2920	DetectaGene™ Green CMFDG *lacZ* Gene Expression Kit	1 kit
D-6488	9*H*-(1,3-dichloro-9,9-dimethylacridin-2-one-7-yl) β-D-galactopyranoside (DDAO galactoside)	5 mg
D-7519	*N*-(4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-*s*-indacene-3-dodecanoyl)sphingosyl 1-β-D-galactopyranoside (BODIPY® FL C_{12}-galactocerebroside)	25 µg
D-7547	*N*-(4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-*s*-indacene-3-dodecanoyl)sphingosyl 1-β-D-glucopyranoside (BODIPY® FL C_{12}-glucocerebroside)	250 µg
D-7548	*N*-(4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-*s*-indacene-3-pentanoyl)sphingosyl 1-β-D-glucopyranoside (BODIPY® FL C_5-glucocerebroside)	250 µg

D-13951	*N*-(4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-*s*-indacene-3-pentanoyl)sphingosyl 1-β-D-lactoside (BODIPY® FL C_5-lactosylceramide)	25 µg
D-11950	6,8-difluoro-4-heptadecylumbelliferyl β-D-galactopyranoside (C_{17}DiFUG)	5 mg
D-11945	6,8-difluoro-4-methylumbelliferyl β-D-galactopyranoside (DiFMUG)	10 mg
D-11951	6,8-difluoro-4-methylumbelliferyl β-D-glucuronide, lithium salt (DiFMUGlcU)	5 mg
D-2893	5-dodecanoylaminofluorescein di-β-D-galactopyranoside (C_{12}FDG)	5 mg
E-22011	ELF® 97 chitinase/*N*-acetylglucosaminidase substrate (ELF® 97 *N*-acetylglucosaminide; ELF® 97 NAG)	5 mg
E-6587	ELF® 97 β-D-glucuronidase substrate (ELF® 97 β-D-glucuronide)	5 mg
E-11954	EnzChek® Amylase Assay Kit *1000 assays*	1 kit
E-22013	EnzChek® Lysozyme Assay Kit *400 assays*	1 kit
F-1930	FluoReporter® *lacZ* Flow Cytometry Kit *50 assays*	1 kit
F-1931	FluoReporter® *lacZ* Flow Cytometry Kit *250 assays*	1 kit
F-2905	FluoReporter® *lacZ*/Galactosidase Quantitation Kit *1000 assays*	1 kit
F-1179	fluorescein di-β-D-galactopyranoside (FDG)	5 mg
F-2881	fluorescein di-β-D-glucopyranoside (FDGlu)	5 mg
F-2915	fluorescein di-β-D-glucuronide (FDGlcU)	5 mg
I-2904	ImaGene Green™ C_{12}FDG *lacZ* Gene Expression Kit	1 kit
I-2908	ImaGene Green™ C_{12}FDGlcU GUS Gene Expression Kit	1 kit
I-2906	ImaGene Red™ C_{12}RG *lacZ* Gene Expression Kit	1 kit
I-14402	Influx™ pinocytic cell-loading reagent *makes 10 x 5 mL*	1 set
M-1489	4-methylumbelliferyl β-D-galactopyranoside (MUG)	1 g
M-1490	4-methylumbelliferyl β-D-glucuronide (MUGlcU)	100 mg
O-2892	5-octanoylaminofluorescein di-β-D-galactopyranoside (C_8FDG)	5 mg
P-11948	5-(pentafluorobenzoylamino)fluorescein di-β-D-galactopyranoside (PFB-FDG)	5 mg
P-11947	5-(pentafluorobenzoylamino)fluorescein di-β-D-glucopyranoside (PFB-FDGlu)	5 mg
P-11949	5-(pentafluorobenzoylamino)fluorescein di-β-D-glucuronide (PFB-FDGlcU)	5 mg
P-1692	phenylethyl β-D-thiogalactopyranoside (PETG)	10 mg
R-1159	resorufin β-D-galactopyranoside	25 mg
S-931	streptavidin, β-galactosidase conjugate	1 mg
T-658	β-trifluoromethylumbelliferyl β-D-glucuronide	25 mg

* * * * *

Data Table — 10.2 Detecting Glycosidases

Cat #	MW	St rage	S luble	Abs	EC	Em	S lvent	Pr duct	N tes

A-12222	257.25	FF,D,A	DMSO	280	6,000	none	pH 8	R-363 (&item=363">see data)	1
A-22010	713.65	F,D	DMSO	289	5,500	none	MeOH	see Notes	2
B-1689	408.63	F,D	DMSO	290	4,900	none	H_2O	see Notes	3
B-1690	408.63	F,D	DMSO	290	4,900	none	H_2O	see Notes	3
B-1691	521.79	F,D	pH >6	292	4,800	none	MeOH	see Notes	3
B-8408	521.79	F,D	pH >6	294	4,600	none	pH 7	see Notes	4
B-22015	408.63	F,D	DMSO	290	4,900	none	H_2O	see Notes	3
C-1488	368.30	F,D	pH >6, DMSO	330	16,000	396	pH 8	H-185 (&item=185">see data)	5
C-1492	458.46	F,D	pH >6	329	18,000	395	pH 7	H-185 (&item=185">see data)	5
C-6541	700.52	F,D	pH >5, DMSO	282	8,700	none	MeOH	see Notes	6
C-11946	408.74	F,D,L	DMSO, H_2O	284	8,000	none	pH 9	C-12881 (&item=12881">see data)	
D-2893	853.92	F,D	DMSO	289	6,000	none	MeOH	D-109 (&item=109">see data)	
D-2920	705.07	F,L	see Notes	273	4,800	none	MeOH	see Notes	7, 8, 9
D-6486	484.29	F,D,L	DMSO	463	26,000	607	MeOH	H-6482 (&item=6482">see data)	
D-6488	470.31	F,D,L	DMSO	465	24,000	608	pH 7	H-6482 (&item=6482">see data)	
D-7519	861.96	FF,D,L	DMSO, EtOH	505	85,000	511	MeOH	see Notes	10, 11
D-7547	861.96	FF,D,L	DMSO, EtOH	505	85,000	511	MeOH	see Notes	10, 11
D-7548	763.77	FF,D,L	DMSO, EtOH	505	85,000	511	MeOH	see Notes	10, 11
D-11945	374.29	F,D	DMSO, H_2O	313	6,900	see Notes	pH 9	D-6566 (&item=6566">see data)	5
D-11950	598.72	F,D	DMSO	314	6,400	none	MeCN	D-12760 (see data)	
D-11951	394.21	F,D	H_2O	313	6,200	see Notes	pH 9	D-6566 (&item=6566">see	5

								data)	
D-11952	374.29	F,D	DMSO	313	7,300	see Notes	pH 9	D-6566 (see data)	5
D-11953	598.72	F,D	DMSO	314	7,000	none	MeCN	D-12760 (see data)	
D-13951	925.91	FF,D,L	DMSO, EtOH	505	80,000	511	MeOH	see Notes	10, 11
E-6587	483.26	F,D,L	DMSO, H_2O	302	14,000	see Notes	MeOH	E-6578 (&item=6578">see data)	5
E-22011	511.34	F,D,L	DMSO	290	11,000	see Notes	DMSO	E-6578 (see data)	5
F-1179	656.60	F,D	DMSO	273	6,400	none	MeOH	F-1300 (see data)	12
F-2881	656.60	F,D	DMSO	272	6,200	none	MeOH	F-1300 (see data)	
F-2905	368.30	F,D,L	pH 7	330	16,000	396	pH 8	H-185 (&item=185">see data)	5, 9, 13
F-2915	684.56	F,D	pH >6, DMSO	272	5,700	none	MeOH	F-1300 (&item=1300">see data)	
I-2904	853.92	F,D,L	DMSO	289	6,000	none	MeOH	D-109 (&item=109">see data)	9, 13
I-2906	543.66	F,D,L	see Notes	448	20,000	none	MeOH	see Notes	9, 14, 15
I-2908	881.88	F,D	see Notes	290	5,400	none	MeOH	D-109 (see data)	7, 9
I-6621	238.30	D	DMSO, H_2O	<300		none			
M-1489	338.31	D	DMSO, H_2O	316	14,000	376	pH 9	H-189 (&item=189">see data)	5
M-1490	352.30	F,D	pH >6	316	12,000	375	pH 9	H-189 (&item=189">see data)	5
O-2892	797.81	F,D	DMSO	289	5,500	none	MeOH	see Notes	2
P-1692	300.37	F,D	DMSO, H_2O	<300		none			
P-11947	865.67	F,D	DMSO	260	26,000	none	MeOH	P-12925 (&item=12925">see data)	
P-11948	865.67	F,D	DMSO	260	25,000	none	MeOH	P-12925 (&item=12925">see data)	
P-11949	893.64	F,D	pH >6, DMSO	260	21,000	none	MeOH	P-12925 (&item=12925">see	

								data)	
R-1159	375.33	F,D,L	DMSO	469	19,000	none	pH 9	R-363 (&item=363">see data)	
R-1161	427.41	F,D,L	pH >6	479	17,000	none	pH 8	R-363 (&item=363">see data)	
T-657	392.28	F,D	DMSO	325	13,000	410	pH 9	T-659 (&item=659">see data)	5
T-658	406.27	F,D	pH >6	325	11,000	410	pH 8	T-659 (&item=659">see data)	5

Notes

1. This substrate is used for peroxidase-coupled detection in our Amplex Red Assay Kits.
2. Enzymatic cleavage of this substrate yields a 5-acylaminofluorescein derivative with spectroscopic properties similar to D-109.
3. Enzymatic cleavage of this substrate yields a water-insoluble, blue-colored indigo dye (Abs ~615 nm).
4. Enzymatic cleavage of this substrate yields a water-insoluble, magenta-colored indigo dye (Abs ~565 nm).
5. Fluorescence of the unhydrolyzed substrate is very weak.
6. Product of *N*-acetyl-β-D-glucosaminidase hydrolysis is 6'-(*O*-carboxymethyl)-2',7'-dichlorofluorescein. Abs = 489 nm (EC = 5,600 $cm^{-1}M^{-1}$) in MeOH (Chem Pharm Bull 41, 1513 (1993)).
7. This product is packaged as a solution in 1:1 (v/v) DMSO/H_2O.
8. Enzymatic cleavage of this substrate yields 5-chloromethylfluorescein, with spectroscopic properties similar to C-1904.
9. Data represent the substrate component of this kit.
10. Em for BODIPY FL sphingolipids shifts to ~620 nm when high concentrations of the probe (>5 mol %) are incorporated in lipid mixtures (J Cell Biol 113, 1267 (1991)).
11. Enzymatic cleavage of this product yields a fluorescent ceramide or glycosylceramide with unchanged spectral properties.
12. F-1179 is soluble at 1 mM in water, but it is best to prepare a stock solution in DMSO.
13. This product is supplied as a ready-made solution in the solvent indicated under "Soluble."
14. This product is packaged as a solution in 7:3 (v/v) DMSO/EtOH.
15. Enzymatic cleavage of this substrate yields 2-dodecylresorufin, Abs = 578 nm (EC = 69,000 $cm^{-1}M^{-1}$), Em = 597 nm in MeOH.

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Section 10.3 — Detecting Enzymes that Metabolize Phosphates and Polyphosphates

Cells utilize a wide variety of phosphate and polyphosphate esters as enzyme substrates, second messengers, membrane structural components and vital energy reservoirs. This section includes an assortment of reagents and methods for detecting the metabolism of phosphate esters. Our diverse array of fluorogenic and chromogenic substrates include substrates for phosphatases, as well as reagents to measure the activity of enzymes such as ATPases, GTPases and DNA and RNA polymerases. In addition, we have several nucleotide analogs and substrates for phosphodiesterases and phospholipases that are described in Section 18.3 and Section 18.4, respectively.

By far the largest group of chromogenic and fluorogenic substrates for phosphate-ester metabolizing enzymes are those for simple phosphatases such as alkaline and acid phosphatase, both of which hydrolyze phosphate monoesters to an alcohol and inorganic phosphate. Conjugates of calf intestinal alkaline phosphatase are extensively used as secondary detection reagents in ELISAs,[1] immunohistochemical techniques [2] and Northern, Southern and Western blot analyses (Section 8.5, Section 9.4). In addition, phosphatases serve as enzyme markers, allowing researchers to identify primordial germ cells,[3] to distinguish subpopulations of bone marrow stromal cells [4] and to investigate *in vitro* differentiation in carcinoma cell lines.[5–7] *P ALP-1*, the gene for human placental alkaline phosphatase, has been used as a eukaryotic reporter gene that is superior to *lacZ* for lineage studies in murine retina.[8,9] This gene has also been engineered to produce a secreted alkaline phosphatase (SEAP), allowing quantitation of gene expression without disrupting the cells.[10]

Molecular Probes supplies the best phosphatase substrates and assay kits for a wide variety of applications:

- Fluorescein diphosphate (F-2999) — probably the most sensitive substrate available for alkaline phosphatase activity measurements.
- DDAO phosphate, a long-wavelength, dual-purpose phosphatase substrate for both solution-based assays and amplified detection of specific targets on blots (Section 9.4).
- ELF 97 phosphate, an acid and alkaline phosphatase substrate whose hydrolysis immediately yields a green-fluorescent precipitate at the sites of endogenous phosphatase activity in cells. ELF 97 phosphate is also utilized in our Enzyme-Labeled Fluorescence technology for immunostaining and fluorescence *in situ* hybridization applications (Section 6.3).
- DiFMUP, which is available as a standalone reagent (D-6567, D-22065) and as a component of our EnzChek Acid Phosphatase Assay Kit (E-12020). The RediPlate 96 and RediPlate 384 EnzChek Tyrosine Phosphatase Assay Kits (R-22067, R-22068) and the RediPlate 96 EnzChek Serine/Threonine Phosphatase Assay Kit (R-33700) utilize the DiFMUP substrate for assaying tyrosine phosphatase activity and serine/threonine phosphatase activity, respectively, and for screening phosphatase inhibitors in a convenient high-throughput format.
- 5-Bromo-4-chloro-3-indolyl phosphate (BCIP, B-6492), a chromogenic phosphatase substrate that is extensively used in various histological and molecular biology techniques.
- BODIPY FL ATP-γ-S (A-22184) and BODIPY FL GTP-γ-S (G-22183) thioesters, which are important substrates for Fhit, a member of the histidine triad superfamily of nucleotide-binding proteins.[11]
- The P_iPer and EnzChek Phosphate and EnzChek Pyrophosphate Assay Kits — unique products that can assay a wide variety of phosphatases, including ATPases and GTPases, that cannot be assayed with the typical fluorogenic or chromogenic phosphatase substrates.
- The BOLD APB chemiluminescent substrate [12] (B-21901), whose sensitivity for detection of

alkaline phosphatase–labeled probes on blots we find to be superior to that of other commercially available chemiluminescent phosphatase substrates (Section 9.4, Figure 9.63).

Phosphatase Substrates Yielding Soluble Fluorescent Products

Fluorescein Diphosphate

First described in 1963,[13] fluorescein diphosphate (FDP, F-2999) is perhaps the most sensitive fluorogenic phosphatase substrate available. The colorless and nonfluorescent FDP reagent is hydrolyzed to fluorescein (F-1300, Section 10.1), which exhibits superior spectral properties (ϵ ~90,000 $cm^{-1}M^{-1}$, quantum yield ~0.92; Figure 1.54). We have succeeded in preparing a highly purified FDP and find it to be an excellent substrate for alkaline phosphatase in ELISAs, providing detection limits at least 50 times lower than those obtained with the chromogenic 4-nitrophenyl phosphate.[14] The relatively high pH required to monitor alkaline phosphatase activity is advantageous because it also enhances fluorescein's fluorescence. FDP has been used for a diverse set of applications, including:

- Measuring endogenous phosphatase in an assay for cell adhesion and migration that is reported to be as sensitive as ^{51}Cr-release assays [15]
- Quantitating the effect of inhibitors on tyrosine phosphatases [16–18]
- Detecting alkaline phosphatase immobilized on microspheres in an optical sensor array [19]
- Assaying diarrheic shellfish toxins in a microplate assay [20]
- Assaying alkaline phosphatase in a microfluidic device [21]

Dimethylacridinone (DDAO) Phosphate

Our 7-hydroxy-9*H*-(1,3-dichloro-9,9-dimethylacridin-2-one (DDAO phosphate, D-6487; Figure 10.27) phosphatase substrate yields a hydrolysis product that is efficiently excited by the 633 nm spectral line of the He–Ne laser to produce bright red fluorescence with absorption/emission maxima of ~646/659 nm [22] (Figure 10.6). Although the substrate itself is fluorescent, the difference between the substrate's excitation maximum and that of the product is over 200 nm (Figure 10.12), allowing the two species to be easily distinguished. Like other DDAO-based substrates, DDAO phosphate has good water solubility, a low K_M and a high turnover rate, making it particularly useful for both fluorescence- and absorption-based microplate assays.[23] Our Pro-Q Western Blot Stain Kits, Pro-Q Glycoprotein Blot Stain Kits and some of our DyeChrome Western Blot Stain Kits (Table 9.6) utilize DDAO phosphate in combination with alkaline phosphatase conjugates for the ultrasensitive detection of proteins on blots. Unlike chemiluminescent assays, the Pro-Q and DyeChrome Blot Stain Kits give permanent staining of the blots. DDAO phosphate and the Amplex Gold peroxidase substrate are used in combination with the SYPRO Ruby Blot Stain in the DyeChrome Double Western Blot Stain Kit (D-21887) for the simultaneous detection of two specific proteins and total proteins on Western blots. These kits are described in detail in Section 9.4.

Methylumbelliferyl Phosphate (MUP) and Difluorinated Methylumbelliferyl Phosphate (DiFMUP)

We offer 4-methylumbelliferyl phosphate (MUP), probably the most widely used fluorogenic substrate for alkaline phosphatase detection, as either its free acid (M-6491) or dicyclohexylammonium salt (M-8425). MUP has been used for a variety of ELISA protocols [24] in which the relatively high pH optimum of alkaline phosphatase permits continuous detection of the rate of formation of 4-methylumbelliferone (7-hydroxy-4-methylcoumarin, H-189, Section 10.1). MUP has also been used to count cells based on their alkaline phosphatase activity,[25] to detect PCR amplification products [26,27] and to identify and characterize bacteria.[28,29]

Our patented 6,8-difluoro-4-methylumbelliferyl phosphate (DiFMUP, D-6567, D-22065; Figure 10.28) exhibits extraordinary spectral properties that are proving advantageous for the assay of both acid and alkaline phosphatase activity and is probably the best general substrate available for measuring the activity of the protein phosphatases that are important for high-throughput screening applications under physiological conditions.[30,31] The hydrolysis product of DiFMUP — 6,8-difluoro-4-methylumbelliferone (6,8-difluoro-7-hydroxy-4-methylcoumarin, D-6566; Section 10.1; Figure 10.29) — exhibits both a lower pK_a (4.9 versus 7.8, Figure 1.101) and a higher fluorescence quantum yield (0.89 versus 0.63) than the hydrolysis product of MUP.[32] The low pK_a of its hydrolysis product makes DiFMUP a sensitive substrate for the continuous assay of acid phosphatases (Figure 10.30, Figure 10.31), which is not possible with MUP because its fluorescence must be measured at alkaline pH (pH >9). Furthermore, with its high fluorescence quantum yield, DiFMUP increases the sensitivity of both acid and alkaline phosphatase measurements. As with our fluorinated fluorescein derivatives (Oregon Green dyes, Section 1.5), fluorination renders the methylumbelliferone fluorophore much less susceptible to photobleaching, yet does not significantly affect the extinction coefficient or excitation/emission maxima. DiFMUP is available as a single 5 mg vial (D-6567) or as a set of 10 vials, each containing 10 mg of the substrate (D-22065) for high-throughput screening applications. DiFMUP is also used in our EnzChek Acid Phosphatase Detection Kit (E-12020), our RediPlate 96 and RediPlate 384 EnzChek Tyrosine Phosphatase Assay Kits (R-22067, R-22068) and our RediPlate 96 EnzChek Serine/Threonine Phosphatase Assay Kit (R-33700), which are described below with our other phosphatase assay kits.

ELF 97 Phosphate — A Phosphatase Substrate That Yields a Fluorescent Precipitate

Molecular Probes' scientists have made a major breakthrough in the development of substrates that yield fluorescent precipitates at the site of enzymatic activity — a patented process we call Enzyme-Labeled Fluorescence (ELF) [33] (Figure 10.32, Figure 10.33). Our first product in this line was the ELF 97 phosphatase substrate (ELF 97 phosphate, E-6588, E-6589). Upon enzymatic cleavage (Figure 6.15), this weakly blue-fluorescent substrate yields an extremely photostable (Figure 6.18) green-fluorescent precipitate that is up to 40 times brighter than the signal achieved when using either directly labeled fluorescent hybridization probes or fluorescent secondary detection methods in comparable applications.[34–36] ELF 97 phosphate can be used over a wide pH range to selectively detect either acid- or alkaline-phosphatase activity. Although optimally excited with UV light (Figure 6.17), the ELF 97 alcohol precipitate can also reportedly be excited with the 488 nm spectral line of the argon-ion laser, making it compatible with flow cytometry and confocal laser-scanning microscopy.[37] Using ELF 97 phosphate and confocal laser-scanning microscopy, researchers have developed a semi-automated method for analyzing the position within a regenerating newt limb of transfected cells expressing the secreted alkaline phosphatase reporter gene.[37] Kits based on our ELF 97 phosphate include the:

- ELF 97 mRNA *In Situ* Hybridization Kits [38] (E-6604, E-6605; Figure 6.21, Figure 8.86)
- ELF 97 Cytological Labeling Kit (E-6603, Figure 6.23, Figure 6.26)
- ELF 97 Immunohistochemistry Kit (E-6600, Figure 6.2)
- ELF 97 Endogenous Phosphatase Detection Kit (E-6601, Figure 6.29, Figure 10.34)

These kits and their contents are described in greater detail in Section 6.3. ELF 97 phosphate is also available separately as a 5 mM solution in water containing sodium azide (E-6589) or in a 0.2 µm–filtered solution (E-6588). Filtration of ELF 97 phosphate through an ELF spin filter (E-6606) is recommended before use. We have found that enzyme-catalyzed precipitation is often improved by incorporating a trace amount of the hydrolysis product — the yellow-green–fluorescent ELF 97 alcohol — in the detection buffer. The ELF 97 alcohol is available in a concentrated solution in DMSO (E-6578, Section 10.1).

The ELF 97 phosphate substrate can be used to detect endogenous phosphatase activity (Figure

10.35) or in combination with alkaline phosphatase conjugates of secondary antibodies (Section 7.3), streptavidin (Section 7.6), concanavalin A (Section 7.7) or other alkaline phosphatase conjugates for signal amplification. A particularly easy means of preparing alkaline phosphatase–labeled antibodies utilizes our exclusive Zenon Alkaline Phosphatase Antibody Labeling Kits (Section 7.2, Table 7.1). With these kits it is possible to rapidly and quantitatively label even extremely small (sub-microgram) quantities of an antibody. Following labeling of the target, the alkaline phosphatase activity of the complex can be detected with the ELF 97 phosphatase substrate or any of the other phosphatase substrates in this section. We have directly detected the alkaline phosphatase enzyme with an antibody to the protein (Figure 10.36).

A different ELF phosphatase substrate — ELF 39 phosphate — is used to detect alkaline phosphatase–conjugated secondary antibodies or streptavidin in three of our DyeChrome Western Blot Stain Kits (Section 9.4, Table 9.6).

Our patented ELF technology and products [33] are offered for research purposes only. Molecular Probes welcomes inquiries about Licensing these products for resale or other commercial uses.

BODIPY FL ATP-γ-*S* and BODIPY FL GTP-γ-*S* Thioesters: Substrates for the Fhit Nucleotide-Binding Protein

The patented BODIPY FL ATP-γ-*S* (A-22184) and BODIPY FL GTP-γ-*S* (G-22183) thioesters are important substrates for Fhit, a member of the histidine triad superfamily of nucleotide-binding proteins.[11] This enzyme binds and cleaves diadenosine polyphosphates and functions as a tumor suppressor in human epithelial cancers.[39,40] In addition to their use for screening potential Fhit inhibitors, these BODIPY FL nucleotides should also be important for studying other nucleotide-binding proteins because they bind to ATP- or GTP-binding sites but are not metabolized (Section 18.3). As with other fluorescent nucleotides, the fluorescence polarization properties of the bound nucleotide should differ from that of the free nucleotide. The fluorescence of BODIPY FL GTP-γ-*S* thioester is quenched ~90% relative to that of the free dye (Figure 18.18) but is recovered upon binding to at least some G-proteins.[41,42]

Chromogenic Phosphatase Substrate

BCIP

5-Bromo-4-chloro-3-indolyl phosphate (BCIP, B-6492) is commonly used with a number of different chromogens in various histological and molecular biology techniques (Section 8.4, Section 9.4). Hydrolysis of this indolyl phosphate, followed by oxidation, produces a blue-colored precipitate at the site of enzymatic activity.

NBT: A Co-Precipitant for the BCIP Reaction

Nitro blue tetrazolium (NBT, N-6495) is the most commonly used electron-transfer agent and co-precipitant for the BCIP reaction, forming a dark blue, precisely localized precipitate in the presence of alkaline phosphatase [43,44] (Figure 9.64, Figure 10.37). Our BCIP and NBT reagents are already priced very competitively, but our NBT/BCIP Reagent Kit (N-6547) offers 1 g samples of both the sodium salt of BCIP and NBT at an even lower price.

Kits for Detecting Phosphatases, Polymerases and Nucleases

Molecular Probes has developed some unique products for following the activity of phosphatases, polymerases and nucleases. Our patented P_iPer Phosphate and P_iPer Pyrophosphate Assay Kits provide ultrasensitive assays for free phosphate and pyrophosphate, respectively, through the formation of resorufin, which can be detected either fluorometrically or spectrophotometrically. Our EnzChek Phosphate and EnzChek Pyrophosphate Assay Kits provide colorimetric assays for

inorganic phosphate and pyrophosphate, respectively. Both the P_iPer and the EnzChek Phosphate Assay Kits permit continuous measurement of the activity of ATPases, GTPases, phosphatases, nucleotidases, kinases and a number of enzymes that produce or consume inorganic phosphate. The P_iPer Kit is more sensitive and better suited for high-throughput studies in microplates, but requires an additional enzymatic step, whereas the EnzChek Kit is less sensitive but has a simpler protocol. In both the P_iPer and the EnzChek Pyrophosphate Assay Kits, the phosphate assay is coupled with the enzyme, pyrophosphatase, in order to monitor the activity of pyrophosphate-producing enzymes such as DNA- and RNA-polymerases, adenylate cyclase and guanylate cyclase.[45,46] In addition to these kits, our EnzChek Acid Phosphatase Detection Kit employs the fluorogenic substrate DiFMUP for the continuous assay of all phosphatases, including acid phosphatases, which are not compatible with assays based on MUP because its fluorescence must be measured at alkaline pH. The RediPlate 96 and RediPlate 384 EnzChek Tyrosine Phosphatase Assay Kits (R-22067, R-22068, see below) and the RediPlate 96 EnzChek Serine/Threonine Phosphatase Assay Kit (R-33700) utilize DiFMUP for assaying tyrosine phosphatase activity and serine/threonine phosphatase activity, respectively, and for screening phosphatase inhibitors in a convenient high-throughput format.

P_iPer Phosphate Assay Kit

Molecular Probes' patented P_iPer Phosphate Assay Kit [47] (P-22061) provides an ultrasensitive assay that detects free phosphate in solution through the formation of the fluorescent product resorufin. Because resorufin also has strong absorption, the assay can be performed either fluorometrically or spectrophotometrically. This kit can be used to detect inorganic phosphate (P_i) in a variety of samples or to monitor the kinetics of phosphate release by a variety of enzymes, including ATPases, GTPases, 5'-nucleotidase, protein phosphatases, acid and alkaline phosphatases and phosphorylase kinase. Furthermore, the assay can be modified to detect virtually any naturally occurring organic phosphate molecule by including an enzyme that can specifically digest the organic phosphate to liberate inorganic phosphate.

In the P_iPer phosphate assay (Figure 10.38), maltose phosphorylase converts maltose (in the presence of P_i) to glucose 1-phosphate and glucose. Then glucose oxidase converts the glucose to gluconolactone and H_2O_2. Finally, with horseradish peroxidase (HRP) as a catalyst, the H_2O_2 reacts with the Amplex Red reagent (10-acetyl-3,7-dihydroxyphenoxazine) to generate resorufin, which has absorption/emission maxima of ~563/587 nm [48,49] (Figure 10.5). The resulting increase in fluorescence or absorption is proportional to the amount of P_i in the sample. This kit can be used to detect as little as 0.2 µM P_i by fluorescence (Figure 10.39) or 0.4 µM P_i by absorption.

Each P_iPer Phosphate Assay Kit contains:

- The Amplex Red reagent
- DMSO and a concentrated reaction buffer
- Recombinant maltose phosphorylase from *E. coli*
- Maltose
- Glucose oxidase from *A. niger*
- Horseradish peroxidase (HRP)
- A phosphate standard
- Hydrogen peroxide
- A detailed protocol for detecting phosphatase activity

Each kit provides sufficient reagents for approximately 1000 assays using a reaction volume of 100 µL per assay and either a fluorescence or absorbance microplate reader.

P_iPer Pyr ph sphate Assay Kit

Our P_iPer Pyrophosphate Assay Kit (P-22062) provides a sensitive fluorometric or colorimetric method for measuring the inorganic pyrophosphate (PP_i) in experimental samples or for monitoring the kinetics of PP_i release by a variety of enzymes, including DNA and RNA polymerases, adenylate cyclase and *S*-acetyl coenzyme A synthetase. In the P_iPer pyrophosphate assay, inorganic pyrophosphatase hydrolyzes PP_i to two molecules of inorganic phosphate (P_i). The PP_i then enters into the same cascade of reactions as it does in the P_iPer Phosphate Assay Kit (Figure 10.38). In this case, the resulting increase in fluorescence or absorption is proportional to the amount of PP_i in the sample. This kit can be used to detect as little as 0.1 µM PP_i by fluorescence or 0.2 µM PP_i by absorption (Figure 10.40).

Each P_iPer Pyrophosphate Assay Kit contains:

- The Amplex Red reagent
- Dimethylsulfoxide (DMSO) and a concentrated reaction buffer
- Recombinant maltose phosphorylase from *Escherichia coli*
- Maltose
- Glucose oxidase from *Aspergillus niger*
- Horseradish peroxidase (HRP)
- Inorganic pyrophosphatase from baker's yeast
- A pyrophosphate standard
- A detailed protocol for detecting phosphatase activity

Each kit provides sufficient reagents for approximately 1000 assays using a reaction volume of 100 µL per assay and either a fluorescence or absorbance microplate reader.

EnzChek Phosphate Assay Kit

Continuous assay of many phosphate ester–metabolizing enzymes is difficult because suitable substrates are not available. It usually has been necessary to determine inorganic phosphate release using tedious colorimetric assays or radioisotope-based methods. The EnzChek Phosphate Assay Kit (E-6646), which is based on a method originally described by Webb,[50,51] provides a rapid and highly sensitive enzymatic assay for detecting inorganic phosphate through the formation of a chromophoric product (Figure 10.41). This unique spectrophotometric technique permits the continuous assay of ATPase activity, and potentially the activity of many other enzymes such as GTPases and phosphatases that produce inorganic phosphate. Each EnzChek Phosphate Assay Kit contains:

- 2-Amino-6-mercapto-7-methylpurine riboside (MESG)
- Purine nucleoside phosphorylase (PNP)
- A concentrated reaction buffer
- A KH_2PO_4 standard
- A detailed protocol for detecting and quantitating phosphate

In the presence of inorganic phosphate, MESG is enzymatically converted by PNP to ribose 1-phosphate and the chromophoric product 2-amino-6-mercapto-7-methylpurine (Figure 10.41). Although the MESG reagent is somewhat unstable above pH 7, the reaction can be performed over the pH range of 6.5 to 8.5 with the proper controls.[51] This kit contains a sufficient amount of each reagent for about 100 phosphate assays using 1 mL assay volumes and standard cuvettes.

The substrate MESG and the enzyme PNP included in our EnzChek Phosphate Assay Kit have

already been adapted for monitoring the kinetics of phosphate release by:

- Actin-activated myosin ATPase [51,52]
- Actin polymerization [53]
- Aminoacyl-tRNA synthetase [54]
- Aspartate transcarbamylase [50,55]
- ArsA ATPase, the catalytic subunit of the arsenite pump [56]
- Dethiobiotin synthetase [57]
- Glycerol kinase [51]
- Glycogen phosphorylase [58]
- GTPases [59–64]
- ATPases [55,65,66]
- *myo*-Inositol monophosphatase [67]
- Phospholysine and phosphohistidine phosphatases [68,69]
- Phosphorylase *a* phosphatase [70]
- Phosphorylase kinase [71]
- Serine phosphatase [72]
- Self-assembly of actin and tubulin [73]
- Autodephosphorylation of CheY, a response regulator that mediates bacterial chemotaxis [74]

Moreover, the EnzChek phosphate assay is sufficiently fast and quantitative to permit stopped-flow kinetic experiments on enzymes that produce phosphate, an important development for mechanistic enzyme studies.[51]

Although this kit is usually used to determine the inorganic phosphate produced by enzymes such as ATPases and GTPases, it can also be used to specifically quantitate inorganic phosphate contamination of reagents and solution, with a detection limit of ~2 µM (~0.2 µg/mL) and an effective range between 2 and 150 µM inorganic phosphate (between 2 and 150 nanomoles phosphate in a 1 mL volume). For example, the assay has been used for the rapid assay of inorganic phosphate in the presence of high concentrations of acid-labile phosphates using a microplate reader.[75] The kit's reagents can also be used as a phosphate "mop" to remove almost all inorganic phosphate from a protein solution.[76]

EnzChek Pyrophosphatase Assay Kit

We have adapted the method provided in our EnzChek Phosphate Assay Kit to permit the sensitive spectrophotometric detection of pyrophosphate, which is converted by the enzyme pyrophosphatase to inorganic phosphate. Because two moles of phosphate are released per mole of pyrophosphate consumed, the sensitivity limit of the EnzChek Pyrophosphatase Assay Kit is 1 µM (~0.2 µg/mL). This assay has been adapted to continuously detect several enzymes that liberate pyrophosphate [77–80] such as aminoacyl-tRNA synthetase,[54] luciferase, cytidylyl transferase [81] and *S*-acetyl coenzyme A synthetase [46] and potentially DNA and RNA polymerases, adenylate cyclase and guanylyl cyclase.[45] Each EnzChek Pyrophosphate Assay Kit (E-6645) contains:

- Inorganic pyrophosphatase
- 2-Amino-6-mercapto-7-methylpurine riboside (MESG)
- Purine nucleoside phosphorylase (PNP)
- A concentrated reaction buffer
- A $Na_2P_2O_7$ standard
- A detailed protocol for detecting and quantitating pyrophosphate

The kit contains a sufficient amount of each reagent for about 100 pyrophosphate assays using 1 mL assay volumes and standard cuvettes.

C umarin-Based Pr bes for Detecti n f In rganic Ph sphate

We offer 7-diethylamino-3-((((2-maleimidyl)ethyl)amino)carbonyl)coumarin (MDCC, D-10253; Section 2.3; Figure 2.24) and the corresponding iodoacetamide (IDCC, D-20382, Section 2.3). When conjugated to a mutant phosphate-binding protein, MDCC has proven useful for direct, real-time measurement of inorganic phosphate release during enzymatic reactions.[76,82–86] An IDCC conjugate of a mutant nucleoside diphosphate kinase has been used as a fluorescent sensor of the phosphorylation state of the kinase and to monitor purine nucleoside diphosphate concentrations in real time.[87] MDCC and IDCC are also useful reagents for general labeling of thiols, with bright blue fluorescence.

EnzChek Acid Phosphatase Assay Kit

The EnzChek Acid Phosphatase Assay Kit (E-12020), which includes our patented DiFMUP substrate, can be used to continuously detect phosphatase activity at neutral, basic or moderately acidic pH. Using the assay outlined in the kit's protocol, we have found that DiFMUP is about 100 times more sensitive than MUP for the detection of prostatic acid phosphatase at pH 5.5 (Figure 10.30) and can measure this activity at a pH that is optimal for the enzyme (Figure 10.31). The EnzChek Acid Phosphatase Assay Kit is perfect for the continuous assay of prostatic acid phosphatase, protein phosphatase 1 or almost any other phosphatase that can be assayed with nonprotein-based substrates such as MUP or 4-nitrophenyl phosphate (PNPP). Each kit contains:

- The DiFMUP substrate
- A concentrated reaction buffer
- Acid phosphatase from potato for use as a positive control
- 6,8-Difluoro-7-hydroxy-4-methylcoumarin for use as a reference standard
- A detailed protocol for detecting phosphatase activity

The kit provides a sufficient amount of each reagent for performing approximately 1000 assays, using a reaction volume of 100 µL per assay.

RediPlate 96 and RediPlate 384 EnzChek Tyrosine Phosphatase Assay Kits

Protein tyrosine phosphatases (PTP) represent a large family of enzymes that play a very important role in intra- and intercellular signaling. PTPs work antagonistically with protein tyrosine kinases to regulate signal transduction pathways in response to a variety of signals, including hormones and mitogens.[88–90] Our RediPlate 96 and RediPlate 384 EnzChek Tyrosine Phosphatase Assay Kits (R-22067, R-22068) provide researchers with a sensitive and convenient means to monitor PTP and screen PTP inhibitors for a variety of research areas, including:

- Insulin regulation [91–93]
- Cell proliferation and differentiation [94]
- Axonal outgrowth [95,96]
- Immune response and inflammation [97]
- Angiogenesis [98]

Our tyrosine phosphatase assay is based on our patented 6,8-difluoro-4-methylumbelliferyl phosphate (DiFMUP, D-6567, D-22065), a phosphatase substrate whose hydrolysis product (6,8-difluoro-7-hydroxy-4-methylcoumarin, DiFMU; D-6566, Section 10.1, Figure 10.29) exhibits excitation/emission maxima of ~358/455 nm (Figure 10.2), a low pK_a (~4.9, Figure 1.101) and a high quantum yield (~0.89). Unlike other end-point tyrosine phosphatase assay kits, the RediPlate

96 and RediPlate 384 EnzChek tyrosine phosphatase assay is continuous, allowing researchers to easily measure fluorescence at various time points in order to follow the kinetics of the reaction. Furthermore, the assay is not affected by free phosphate and is compatible with most nonionic detergents. Most importantly, each assay well contains inhibitors to ensure that the assay is selective for tyrosine phosphatases; other phosphatases, including serine/threonine phosphatases, will not hydrolyze DiFMUP under our assay conditions (Figure 10.42).

Each RediPlate 96 EnzChek Tyrosine Phosphatase Assay Kit (R-22067) or RediPlate 384 EnzChek Tyrosine Phosphatase Assay Kit (R-22068) includes one microplate of either 96 or 384 wells, respectively, contained in a resealable foil packet to ensure the integrity of the fluorogenic components, plus a vial of reaction buffer. The first row of wells in the microplate contains a dilution series of the 6,8-difluoro-7-hydroxy-4-methylcoumarin (DiFMU) reference standard for generating a standard curve, and the remaining wells are preloaded with the DiFMUP substrate. Additionally, each RediPlate 96 assay plate has removable lanes that allow researchers to perform only as many assays as required for the experiment (Figure 8.54). The RediPlate 384 EnzChek Tyrosine Phosphatase Assay Kit includes a single 384-well plate for high-throughput screening of tyrosine phosphatase activity. Table 10.3 summarizes our other RediPlate 96 and RediPlate 384 Assay Kits for serine/threonine phosphatase activity (see below), dsDNA quantitation (Section 8.3) and protease activity (Section 10.4). Significant discounts apply to purchases of multiple units of all of our RediPlate products.

RediPlate 96 EnzChek Serine/Threonine Phosphatase Assay Kit

The majority of protein phosphorylation occurs on serine and threonine residues, with <0.01–0.05% on tyrosine residues.[88] Serine/threonine phosphatases represent a large family of enzymes that have been implicated in the regulation of metabolism,[99] transcription,[100] translation,[101] differentiation,[102] cell cycle,[88] cytoskeletal dynamics,[103] oncogenesis [104,105] and signal transduction.[106,107] Molecular Probes' RediPlate 96 EnzChek Serine/Threonine Phosphatase Assay Kit (R-33700) provides a fast, simple and direct fluorescence-based assay for detecting serine/threonine phosphatases and their corresponding modulators and inhibitors.

As with the RediPlate EnzChek Tyrosine Phosphatase Kits, the substrate incorporated in the RediPlate 96 EnzChek Serine/Threonine Phosphatase Assay Kit is our patented DiFMUP, which upon hydrolysis generates DiFMU (D-6566, Section 10.1, Figure 10.29) with excitation/emission maxima of 358/452 nm, a low pK_a (~4.9) and a high quantum yield (~0.89). Inhibitors are included in each assay well to ensure that the assay is selective for serine/threonine phosphatases; under the prescribed assay conditions, other phosphatases, including tyrosine phosphatases, do not significantly react with the substrate. Additional advantages of this Rediplate assay include compatibility with nonionic detergents and insensitivity to free phosphate, resulting in minimal sample processing before analysis.

Each RediPlate 96 EnzChek Serine/Threonine Phosphatase Assay Kit (R-33700) includes on 96-well microplate (contained in a resealable foil packet), a bottle of reaction buffer and several buffer additives. The microplate consists of twelve removable strips, each with eight wells (Figure 8.54). Eleven of the strips (88 wells) are preloaded with the fluorogenic substrate DiFMUP. The remaining strip, marked with blackened tabs, contains a dilution series of the DiFMU reference standard for generating a standard curve. We also offer RediPlate 96 and RediPlate 384 Assay Kits for tyrosine phosphatase activity (see above), dsDNA quantitation (Section 8.3) and protease activity (Section 10.4), which are summarized in Table 10.3. Significant discounts apply to purchases of multiple units of all of our RediPlate products.

ATP Determinati n Kit

Molecular Probes has a convenient ATP Determination Kit (A-22066) for the sensitive bioluminescence-based detection of ATP with recombinant firefly luciferase and its substrate, luciferin. This assay is based on luciferase's absolute requirement for ATP to produce light. In the

presence of Mg^{2+}, luciferase catalyzes the reaction of luciferin, ATP and O_2 to form oxyluciferin, AMP, CO_2, pyrophosphate and ~560 nm light (Figure 15.52). The ATP Determination Kit can also be used in an assay of cell viability and cytotoxicity (Section 15.3).

The luciferin–luciferase bioluminescence assay is extremely sensitive; most luminometers can detect as little as 1 picomole of pre-existing ATP or ATP as it is generated in kinetic systems (Figure 10.43). This sensitivity has led to its widespread use for detecting ATP in various enzymatic reactions, as well as for measuring viable cell number [108] (Section 15.2, Section 15.3) and for detecting low-level bacterial contamination in samples such as blood, milk, urine, soil and sludge.[109–113] The luciferin–luciferase bioluminescence assay has also been used to determine cell proliferation and cytotoxicity in both bacterial [114,115] and mammalian cells,[116,117] and to distinguish cytostatic versus cytocidal potential of anticancer drugs on malignant cell growth.[118]

Each ATP Determination Kit (A-22066) contains:

- Luciferin (5 vials, each containing 3.0 mg)
- Luciferase
- Dithiothreitol (DTT)
- ATP
- A 20× reaction buffer
- A detailed protocol for ATP quantitation

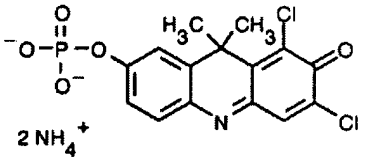
Unlike most other commercially available ATP detection kits, our ATP Determination Kit provides the luciferase and luciferin packaged separately, which enables researchers to optimize the reaction conditions for their particular instruments and samples. The ATP Determination Kit provides a sufficient amount of each reagent to perform 200 ATP assays using 0.5 mL sample volumes or 500 ATP assays using 0.2 mL sample volumes.

1. Methods Mol Biol 32, 461 (1994); **2.** J Clin Microbiol 19, 230 (1984); **3.** Anatomical Record 118, 135 (1954); **4.** J Histochem Cytochem 40, 1059 (1992); **5.** Dev Biol 88, 279 (1981); **6.** Cell 5, 229 (1975); **7.** Proc Natl Acad Sci U S A 70, 3899 (1973); **8.** Biotechniques 14, 818 (1993); **9.** Proc Natl Acad Sci U S A 89, 693 (1992); **10.** Methods Enzymol 216, 362 (1992); **11.** J Biol Chem 275, 4555 (2000); **12.** BOLD is a trademark of Serologicals Corporation; manufactured for Molecular Probes by Serologicals Corporation; patent pending. **13.** Proc Natl Acad Sci USA 50, 1 (1963); **14.** J Immunol Methods 149, 261 (1992); **15.** J Immunol Methods 192, 165 (1996); **16.** J Biol Chem 272, 22472 (1997); **17.** Biochem Pharmacol 54, 721 (1997); **18.** Biochem Pharmacol 54, 703 (1997); **19.** Anal Chem 70, 1242 (1998); **20.** Anal Biochem 248, 258 (1997); **21.** Drug Discov Today HTS Suppl 1, 33 (2000); **22.** DDAO-based substrates are licensed to Molecular Probes under US 4,810,636 and its foreign equivalents; **23.** Toxicon 38, 1833 (2000); **24.** J Immunol Methods 150, 23 (1992); **25.** In Vitro Cell Dev Biol 25, 105 (1989); **26.** Anal Biochem 205, 1 (1992); **27.** Mol Cell Probes 6, 489 (1992); **28.** Zentralbl Bakteriol 280, 476 (1994); **29.** Microbiol Rev 55, 335 (1991); **30.** Anal Biochem 273, 41 (1999); **31.** US 5,830,912; **32.** Bioorg Med Chem Lett 8, 3107 (1998); **33.** US 5,316,906; US 5,443,986 and EP 0,641,351; **34.** Am J Hum Genet 55 (Suppl), A271, abstract #1585 (1994); **35.** FASEB J 8, A1444, abstract #1081 (1994); **36.** J Histochem Cytochem 47, 1443 (1999); **37.** J Histochem Cytochem 44, 559 (1996); **38.** J Histochem Cytochem 45, 345 (1997); **39.** Am J Pathol 156, 419 (2000); **40.** J Natl Cancer Inst 92, 338 (2000); **41.** J Biol Chem 276, 29275 (2001); **42.** Anal Biochem 291, 109 (2001); **43.** Biotechniques 12, 656 (1992); **44.** Histochemistry 58, 203 (1978); **45.** Biochemistry 35, 11013 (1996); **46.** Anal Biochem 243, 41 (1996); **47.** US 6,265,179. **48.** Anal Biochem 253, 162 (1997); **49.** J Immunol Methods 202, 133 (1997); **50.** Anal Biochem 218, 449 (1994); **51.** Proc Natl Acad Sci U S A 89, 4884 (1992); **52.** Biochemistry 36, 11837 (1997); **53.** Biosci Rep 14, 309 (1994); **54.** Nucleic Acids Res 23, 2886 (1995); **55.** Anal Biochem 246, 86 (1997); **56.** J Biol Chem 274, 16153 (1999); **57.** Biochemistry 34, 10976 (1995); **58.** Anal Biochem 221, 348 (1994); **59.** J Biol Chem 275, 25299 (2000); **60.** Biochemistry 37, 5249 (1998); **61.** J Biol Chem 272, 32830 (1997); **62.** Biochemistry 34, 15592 (1995); **63.** Biochem J 287, 555 (1992); **64.** J Biol Chem 272, 21999 (1997); **65.**

Biochemistry 35, 10922 (1996); **66.** Biochemistry 37, 17209 (1998); **67.** Biochem J 307, 585 (1995); **68.** Anal Biochem 222, 14 (1994); **69.** Biochem J 296, 293 (1993); **70.** Anal Biochem 226, 68 (1995); **71.** Anal Biochem 230, 55 (1995); **72.** Biochemistry 33, 2380 (1994); **73.** Biochemistry 35, 12038 (1996); **74.** J Biol Chem 276, 18478 (2001); **75.** Anal Biochem 230, 173 (1995); **76.** Biochemistry 33, 8262 (1994); **77.** J Biol Chem 275, 17962 (2000); **78.** Biochemistry 39, 2297 (2000); **79.** J Biol Chem 273, 16555 (1998); **80.** J Biol Chem 273, 22151 (1998); **81.** Biochemistry 40, 5041 (2001); **82.** Biochemistry 37, 10381 (1998); **83.** Biophys J 74, 3120 (1998); **84.** J Physiol 501, 125 (1997); **85.** FEBS Lett 364, 59 (1995); **86.** The bacterial clone for expressing the mutant phosphate-binding protein is available from Martin Webb, National Institute for Medical Research, London, UK; **87.** Biochemistry 40, 5087 (2001); **88.** Oncogene 19, 6607 (2000); **89.** Trends Cell Biol 11, 258 (2001); **90.** Curr Opin Cell Biol 13, 182 (2001); **91.** Curr Opin Chem Biol 5, 416 (2001); **92.** J Mol Med 78, 473 (2000); **93.** Biochem Pharmacol 60, 877 (2000); **94.** Cell Res 10, 279 (2000); **95.** Curr Opin Neurobiol 11, 95 (2001); **96.** Neuroreport 11, R5 (2000); **97.** Biochem Biophys Res Commun 286, 721 (2001); **98.** Adv Exp Med Biol 476, 35 (2000); **99.** Proc Natl Acad Sci U S A 98, 13710 (2001); **100.** Biochem Biophys Res Commun 285, 1192 (2001); **101.** J Biol Chem 276, 14829 (2001); **102.** J Neurosci Methods 105, 87 (2001); **103.** Eur J Immunol 30, 3422 (2000); **104.** Proc Natl Acad Sci U S A 97, 3207 (2000); **105.** Eur J Biochem 263, 605 (1999); **106.** Eur J Biochem 269, 1060 (2002); **107.** Proc Natl Acad Sci U S A 98, 13613 (2001); **108.** J Biolumin Chemilumin 10, 29 (1995); **109.** Anal Biochem 175, 14 (1988); **110.** Bio/Technology 6, 634 (1988); **111.** J Clin Microbiol 20, 644 (1984); **112.** J Clin Microbiol 18, 521 (1983); **113.** Methods Enzymol 57, 3 (1978); **114.** Biotechnol Bioeng 42, 30 (1993); **115.** J Biolumin Chemilumin 6, 193 (1991); **116.** Biochem J 295, 165 (1993); **117.** J Immunol Methods 160, 81 (1993); **118.** J Natl Cancer Inst 77, 1039 (1986).

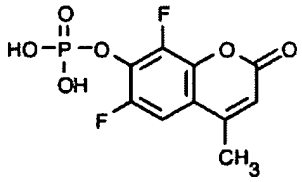
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Figure 10.27 D-6487 9*H*-(1,3-dichloro-9,9-dimethylacridin-2-one-7-yl) phosphate, diammonium salt.



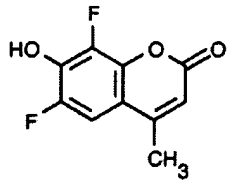
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Figure 10.28 D-6567 6,8-difluoro-4-methylumbelliferyl phosphate.



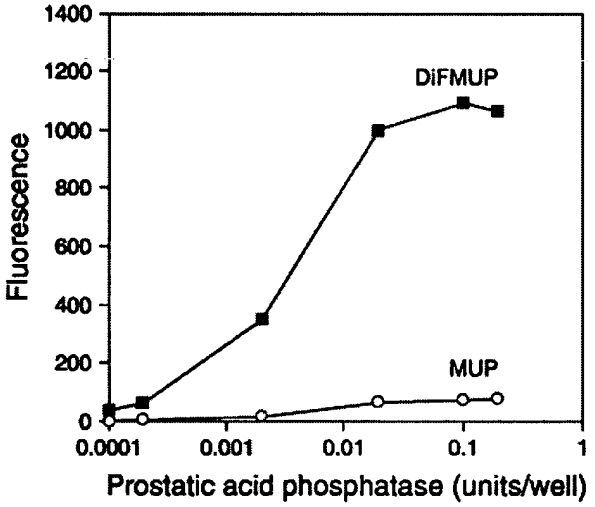
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Figure 10.29 D-6566 6,8-difluoro-7-hydroxy-4-methylcoumarin.



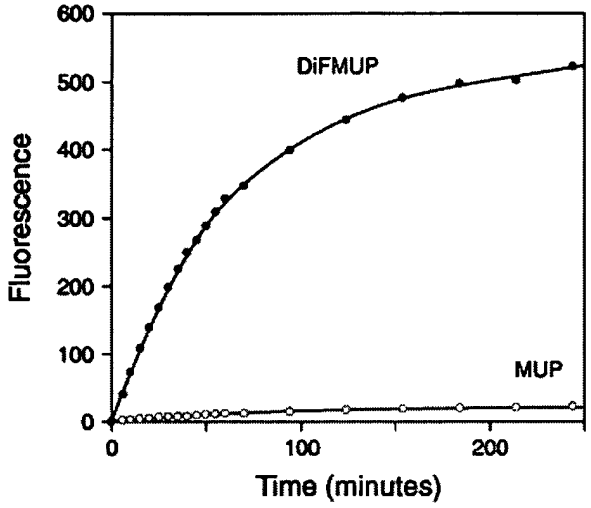
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Figure 10.30 Comparison of DiFMUP (D-6567, D-22065) with MUP (M-6491) for the detection of acid phosphatase activity at pH 5.5. Increasing amounts of prostatic acid phosphatase from human semen were reacted with 100 µM DiFMUP, the substrate in the EnzChek Acid Phosphatase Assay Kit (E-12020), or 100 µM MUP, in 100 mM sodium acetate, pH 5.5, at room temperature. Fluorescence was measured after 60 minutes in a fluorescence-based microplate reader using excitation at 360 ± 20 nm and emission detection at 460 ± 20 nm.



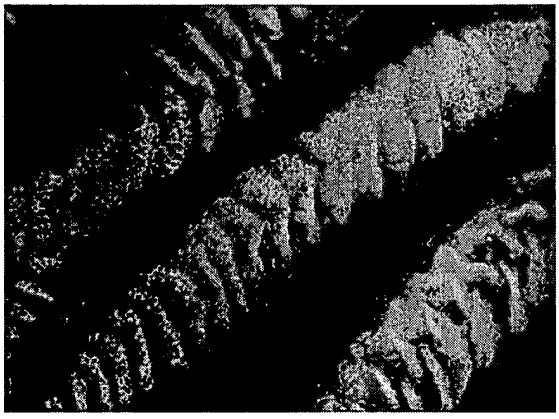
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Figure 10.31 Time course of the reaction of prostatic acid phosphatase with DiFMUP (D-6567, D-22065) and MUP (M-6491). Prostatic acid phosphatase from human semen (0.002 units) was reacted with 100 µM DiFMUP or 100 µM MUP in 100 mM sodium acetate, pH 5.5, at room temperature. Fluorescence was measured at the indicated times in a fluorescence microplate reader using excitation at 360 ± 20 nm and emission detection at 460 ± 20 nm.



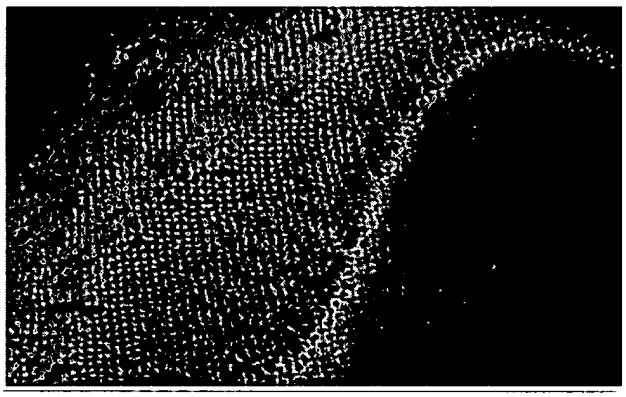
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Figure 10.32 A cryostat section of lightly fixed adult zebrafish gills that have been incubated with the ELF 97 substrate in our ELF 97 Endogenous Phosphatase Detection Kit (E-6601). This kit's novel ELF 97 phosphatase substrate yields a yellow-green–fluorescent precipitate at the site of endogenous phosphatase activity. This staining pattern is identical to that seen when employing the conventional Gomori method for detecting phosphatase activity.



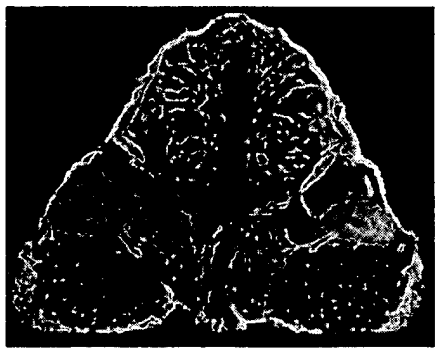
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Figure 10.33 Fixed zebrafish retinal sections stained with FRet 43, an antibody that labels both double cone cells and a subset of bipolar interneurons. The antibody has been developed for visualization with standard enzyme-mediated immunohistochemical techniques using our ELF 97 Immunohistochemistry Kit (E-6600). The ELF 97 alkaline phosphatase substrate in this kit produces an intense yellow-green–fluorescent precipitate at the site of enzymatic activity. The tissue has been sectioned tangentially to reveal the mosaic nature of the zebrafish retina. In this section, the lightly stained round profiles in the lower right are bipolars, whereas the adjacent stained configurations are the double cones, beginning with the synaptic pedicles and progressing through the nuclei, inner segments and outer segments.



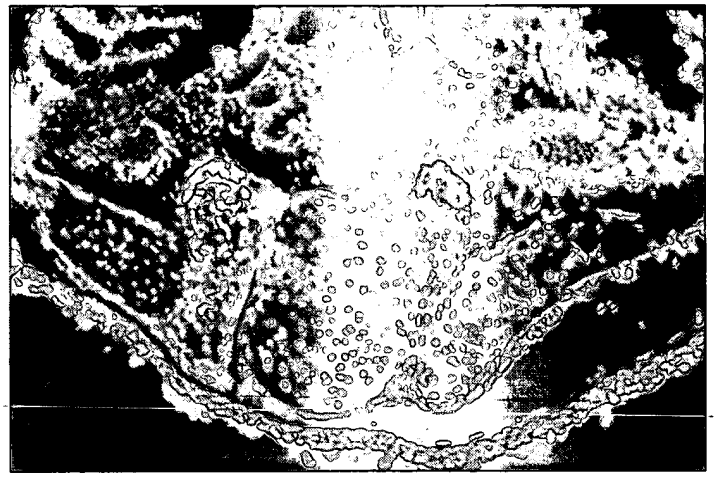
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Figure 10.34 Endogenous alkaline phosphatase activity in the zebrafish brain, localized with the ELF 97 Endogenous Phosphatase Detection Kit (E-6601). Enzymatic cleavage of the ELF 97 phosphatase substrate yields a bright yellow-green–fluorescent precipitate at the site of enzyme activity. The image was acquired with a bandpass filter set appropriate for the ELF alcohol precipitate.



* * * * *

Figure 10.35 Endogenous alkaline phosphatase activity of osteoblast cells in a cartilaginous element of an adult zebrafish head was localized using the ELF 97 Endogenous Phosphatase Detection Kit (E-6601) to stain a cryosection. In addition to the yellow-green fluorescence of the ELF 97 alcohol precipitate, the section was stained with red-fluorescent Texas Red-X wheat germ agglutinin (W-21405) and with the blue-fluorescent Hoechst 33342 nucleic acid stain (H-1399, H-3570, H-21492). The triple-exposure image was acquired using bandpass filter sets appropriate for ELF 97 alcohol, Texas Red dye and DAPI. The image was acquired using a CCD camera controlled by MetaMorph software (Universal Imaging Corporation).



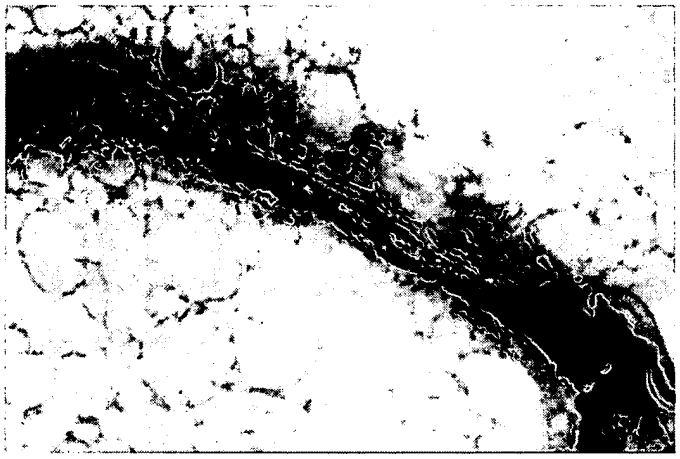
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Figure 10.36 The endogenous alkaline phosphatase enzyme of osteosarcoma cells localized with a mouse anti-rat alkaline phosphatase monoclonal antibody, RBM 211.13, which was visualized with Alexa Fluor 594 goat anti-mouse IgG, $F(ab')_2$ fragments (A-11020). The blue-fluorescent nucleic acid stain Hoechst 33342 (H-1399, H-3570, H-21492) was used as a counterstain to the red fluorescence of the Alexa Fluor 594 secondary antibody. The primary antibody was a gift from Dr. Jane Aubin, University of Toronto. The double-exposure image was acquired using longpass filter sets appropriate for the Texas Red dye and DAPI.



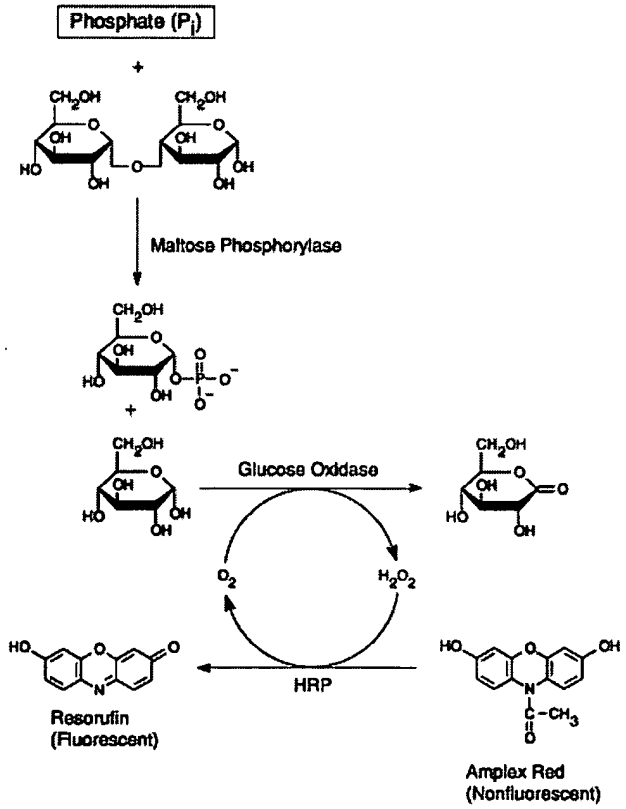
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Figure 10.37 Endogenous alkaline phosphatase activity in a zebrafish ovary cross-section, stained with the BCIP (B-6492) and NBT (N-6495) reagents. These reagents are also available in the NBT/BCIP Reagent Kit (N-6547).



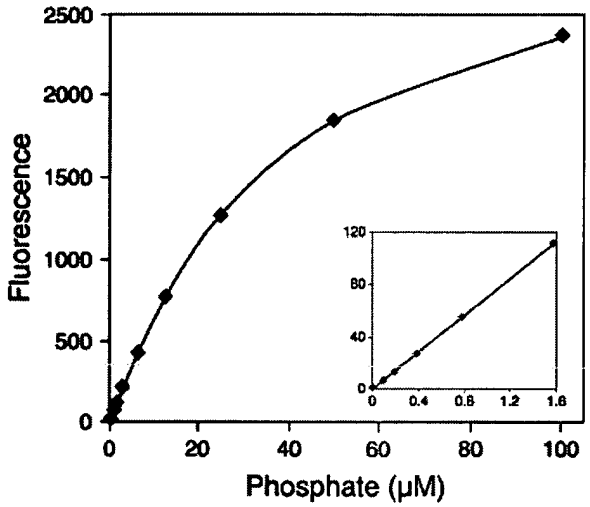
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Figure 10.38 Principle of the P_iPer Phosphate Assay Kit (P-22061). In the presence of inorganic phosphate, maltose phosphorylase converts maltose to glucose 1-phosphate and glucose. Then, glucose oxidase converts the glucose to gluconolactone and H_2O_2. Finally, with horseradish peroxidase (HRP) as a catalyst, the H_2O_2 reacts with the Amplex Red reagent to generate the highly fluorescent resorufin. The resulting increase in fluorescence or absorption is proportional to the amount of P_i in the sample.



* * * * *

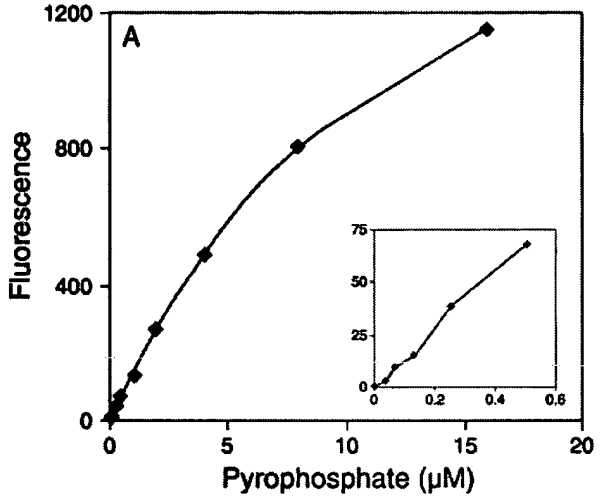
Figure 10.39 Detection of inorganic phosphate using the P_iPer Phosphate Assay Kit (P-22061). Each reaction contained 50 µM Amplex Red reagent, 2 U/mL maltose phosphorylase, 1 mM maltose, 1 U/mL glucose oxidase and 0.2 U/mL HRP in 1x reaction buffer. Reactions were incubated at 37°C. After 60 minutes, fluorescence was measured in a fluorescence microplate reader using excitation at 530 ± 12.5 nm and fluorescence detection at 590 ± 17.5 nm. Data points represent the average of duplicate reactions, and a background value of 43 (arbitrary units) was subtracted from each reading.

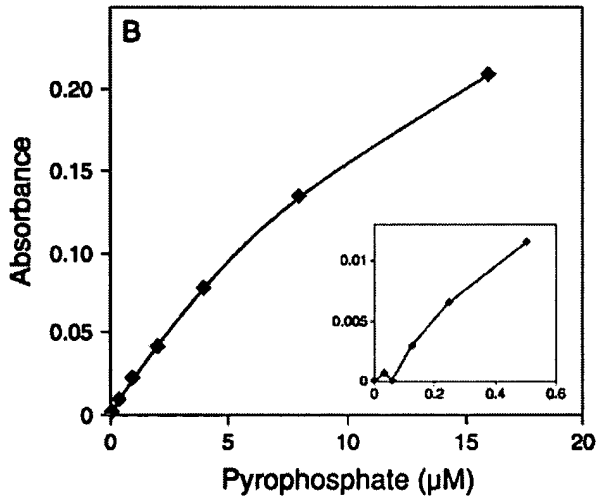


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Figure 10.40 Detection of pyrophosphate using the P_iPer Pyrophosphate Assay Kit (P-22062).

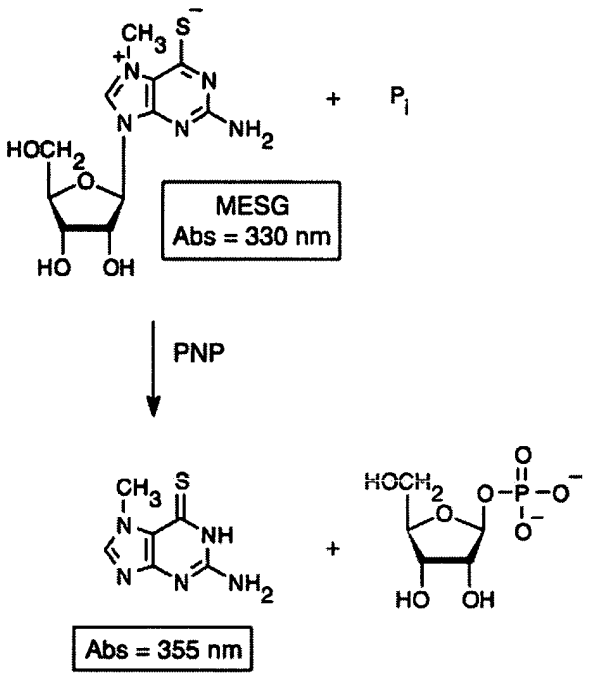
Each reaction contained 50 µM Amplex Red reagent, 0.01 U/mL inorganic pryophosphatase, 2 U/mL maltose phosphorylase, 0.2 mM maltose, 1 U/mL glucose oxidase and 0.2 U/mL HRP in 1× reaction buffer. Reactions were incubated at 37°C. After 60 minutes, fluorescence was measured in a fluorescence-based microplate reader using excitation at 530 ± 12.5 nm and fluorescence detection at 590 ± 17.5 nm **(A)** or absorbance was measured in an absorbance-based microplate reader at 576 ± 5 nm **(B)**. Data points represent the average of duplicate reactions. In panel A, a background value of 78 (arbitrary units) was subtracted from each reading; in panel B, a background absorbance of 0.011 was subtracted from each reading.





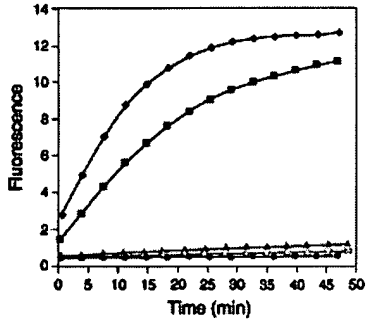
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Figure 10.41 Enzymatic conversion of 2-amino-6-mercapto-7-methylpurine riboside (MESG) to ribose 1-phosphate and 2-amino-6-mercapto-7-methylpurine by purine nucleoside phosphorylase (PNP), reagents supplied in our EnzChek Phosphate Assay Kit (E-6646). The accompanying change in the absorption maximum (Abs) allows quantitation of inorganic phosphate (P_i) consumed in the reaction.



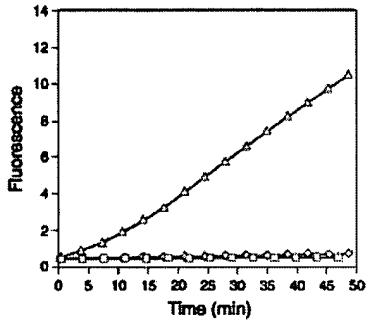
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Figure 10.42 Specificity of the RediPlate 96 and RediPlate 384 EnzChek Tyrosine Phosphatase Assay Kits (R-22067, R-22068) for tyrosine phosphatase. The phosphatases listed in the tables were applied to a RediPlate 96 EnzChek tyrosine phosphatase assay microplate. At the indicated time points, the fluorescence was measured in a fluorescence-based microplate reader using excitation at 355 ± 20 nm and emission at 460 ± 12.5 nm.



Symbol	Enzyme (class)	Enzyme Units*
▲	Acid phosphatase	1 mU/mL
☆	Alkaline phosphatase	1 mU/mL
◆	CD-45 (tyrosine phosphatase)	1 U/mL
	PP2A (ser/thr phosphatase)†	1 U/mL
■	PTP-1B (tyrosine phosphatase)	5 mU/mL

* Enzyme unit (U) definitions are standard definitions for each enzyme. † Serine/threonine phosphatase.

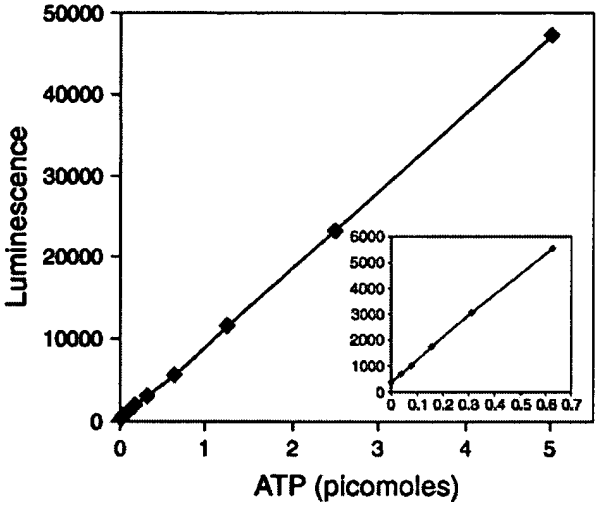


Symbol	Enzyme (class)	Enzyme Units*
□	PP1 (ser/thr phosphatase)†	1 U/mL
◇	PP-2B (ser/thr phosphatase)†	500 U/mL
△	PTPase (tyrosine phosphatase)	1 U/mL

* Enzyme unit (U) definitions are standard definitions for each enzyme. † Serine/threonine phosphatase.

* * * * *

Figure 10.43 Detection of ATP using the ATP Determination Kit (A-22066). Each reaction contained 1.25 µg/mL of firefly luciferase, 50 µM D-luciferin and 1 mM DTT in 1x reaction buffer. Luminescence was measured after a 15-minute incubation.



* * * * *

Figure. Endogenous alkaline phosphatase activity in a zebrafish kidney cross-section, stained with the blue-colored BCIP (B-6492) and NBT (N-6495) reagents. These reagents are also available in the NBT/BCIP Reagent Kit (N-6547).



* * * * *

Product List — 10.3 Detecting Enzymes That Metabolize Phosphates and Polyphosphates

Cat #	Product Name	Unit Size
A-22184	adenosine 5'-*O*-(3-thiotriphosphate), BODIPY® FL thioester, sodium salt (BODIPY® FL ATP-γ-S, thioester) *5 mM in buffer*	50 µL
A-22066	ATP Determination Kit *special packaging* *200-1000 assays*	1 kit
B-21901	BOLD™ APB chemiluminescent substrate *for membrane-based alkaline phosphatase detection* *25 minigel blots*	25 mL
B-6492	5-bromo-4-chloro-3-indolyl phosphate, disodium salt (BCIP, Na)	1 g

D-6487	9*H*-(1,3-dichloro-9,9-dimethylacridin-2-one-7-yl) phosphate, diammonium salt (DDAO phosphate)	5 mg
D-6567	6,8-difluoro-4-methylumbelliferyl phosphate (DiFMUP)	5 mg
D-22065	6,8-difluoro-4-methylumbelliferyl phosphate (DiFMUP) *packaged for high-throughput screening*	10 x 10 mg
E-6603	ELF® 97 Cytological Labeling Kit #2 *with streptavidin, alkaline phosphatase conjugate* *50 assays*	1 kit
E-6601	ELF® 97 Endogenous Phosphatase Detection Kit	1 kit
E-6600	ELF® 97 Immunohistochemistry Kit	1 kit
E-6604	ELF® 97 mRNA *In Situ* Hybridization Kit #1 *50 assays*	1 kit
E-6605	ELF® 97 mRNA *In Situ* Hybridization Kit #2 *with streptavidin, alkaline phosphatase conjugate* *50 assays*	1 kit
E-6589	ELF® 97 phosphatase substrate (ELF® 97 phosphate) *5 mM in water* *contains 2 mM azide*	1 mL
E-6588	ELF® 97 phosphatase substrate (ELF® 97 phosphate) *5 mM in water* *0.2 µm filtered*	1 mL
E-6606	ELF® spin filters *20 filters*	1 box
E-12020	EnzChek® Acid Phosphatase Assay Kit *1000 assays*	1 kit
E-6646	EnzChek® Phosphate Assay Kit *100 assays*	1 kit
E-6645	EnzChek® Pyrophosphate Assay Kit *100 assays*	1 kit
F-2999	fluorescein diphosphate, tetraammonium salt (FDP)	5 mg
G-22183	guanosine 5'-*O*-(3-thiotriphosphate), BODIPY® FL thioester, sodium salt (BODIPY® FL GTP-γ-S, thioester) *5 mM in buffer*	50 µL
M-8425	4-methylumbelliferyl phosphate, dicyclohexylammonium salt, trihydrate (MUP DCA salt)	1 g
M-6491	4-methylumbelliferyl phosphate, free acid (MUP)	1 g
N-6547	NBT/BCIP Reagent Kit	1 kit
N-6495	nitro blue tetrazolium chloride (NBT)	1 g
P-22061	P_iPer™ Phosphate Assay Kit *1000 assays*	1 kit
P-22062	P_iPer™ Pyrophosphate Assay Kit *1000 assays*	1 kit
R-33700	RediPlate™ 96 EnzChek® Serine/Threonine Phosphatase Assay Kit *one 96-well microplate*	1 kit
R-22067	RediPlate™ 96 EnzChek® Tyrosine Phosphatase Assay Kit *one 96-well microplate*	1 kit
R-22068	RediPlate™ 384 EnzChek® Tyrosine Phosphatase Assay Kit *one 384-well microplate*	1 kit

* * * * *

Data Table — 10.3 Detecting Enzymes That Metabolize Phosphates and Polyphosphates

Cat #	MW	St rage	Soluble	Abs	EC	Em	S lvent	Pr duct	N tes
A-22184	878.28	FF,L	H_2O	504	68,000	514	pH 7	see Notes	1, 2, 3
B-6492	370.43	F,D	DMSO, H_2O	292	3,800	none	H_2O	see Notes	4
B-8409	433.63	F,D	DMSO, H_2O	292	5,400	none	pH 7	see Notes	5
D-6487	422.20	F,D,L	DMSO, H_2O	478	26,000	628	pH 7	H-6482 (&item=6482">see data)	
D-6567	292.13	F,D	DMSO, H_2O	320	14,000	385	pH 9	D-6566 (&item=6566">see data)	6
D-22065	292.13	F,D	DMSO, H_2O	320	14,000	385	pH 9	D-6566 (see data)	6
E-6588	431.08	F,D,L	H_2O	289	12,000	see Notes	pH 10	E-6578 (see data)	2, 6
E-6589	431.08	F,L	H_2O	289	12,000	see Notes	pH 10	E-6578 (see data)	2, 6
E-6645	313.33	FF,D	H_2O	332	16,000	none	pH 7	see Notes	7, 8
E-6646	313.33	F,D	H_2O	332	16,000	none	pH 7	see Notes	7, 8
F-2999	560.39	F,D	H_2O	272	5,000	none	MeOH	F-1300 (see data)	
G-22183	894.28	FF,L	H_2O	504	68,000	510	pH 7	see Notes	1, 2, 3, 9
M-6491	256.15	F,D	DMSO, H_2O	319	15,000	383	pH 9	H-189 (see data)	6
M-8425	508.55	F,D	H_2O	318	14,000	385	pH 9	H-189 (see data)	6, 10
N-6495	817.65	D,L	H_2O, DMSO	256	64,000	none	MeOH	see Notes	4
N-6613	371.15	F,D,L	H_2O	310	11,000	none	pH 7	see Notes	10, 11

Notes

1. The molecular weight (MW) of this product is approximate because the degree of hydration and/or salt form has not been conclusively established.
2. This product is supplied as a ready-made solution in the solvent indicated under "Soluble."
3. This compound is a nonhydrolyzable substrate analog.
4. Phosphatase hydrolysis of BCIP (B-6492) is coupled to reduction of NBT (N-6495), yielding a water-insoluble indigo dye (Abs ~615 nm) and a water-insoluble formazan (Abs ~605 nm) respectively.
5. Enzymatic cleavage of this substrate yields a water-insoluble, magenta-colored indigo dye (Abs ~565 nm).

6. Fluorescence of the unhydrolyzed substrate is very weak.
7. Data represent the substrate component of this kit.
8. Enzymatic phosphorylation of this substrate yields 2-amino-6-mercapto-7-methylpurine (Abs = 355 nm) (Proc Natl Acad Sci U S A 89, 4884 (1992)).
9. Fluorescence of BODIPY dye–labeled guanosine derivatives is generally weak due to base-specific intramolecular quenching (Anal Biochem 291, 109 (2001)).
10. MW is for the hydrated form of this product.
11. Enzymatic cleavage of this substrate yields 4-nitrophenol, Abs = 399 nm (EC = 18,000 $cm^{-1}M^{-1}$) in pH 9 buffer.

* * * * *

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Section 10.4 — Detecting Peptidases and Proteases

Peptidases and proteases play essential roles in protein activation, cell regulation and signaling, as well as in the generation of amino acids for protein synthesis or utilization in other metabolic pathways. In general, peptidases cleave shorter peptides, and proteases cleave longer peptides and proteins. A very useful Web site on proteases and protease inhibitors, including an image gallery, is located at the PROLYSIS Home Page.

Depending on their site of cleavage, peptidases can be classified as exopeptidases if they preferentially hydrolyze amino acid residues from the terminus of a peptide, or endopeptidases if they cleave internal peptide bonds.[1] Exopeptidases are further divided into aminopeptidases and carboxypeptidases depending on whether they hydrolyze residues from the amine or the carboxy terminus.

Although the spectral properties of fluorogenic peptidase and protease substrates and their hydrolysis products are easily predictable, the utility of a given substrate for an enzyme depends on the kinetics of hydrolysis by the enzyme, which, in turn, depends on the substrate's concentration and amino acid sequence, as well as on the pH, temperature and presence of cofactors in the medium. For measurements in live cells, the suitability of a particular substrate also hinges on its accessibility to the enzyme and the cellular retention of the hydrolysis product.[2] In addition to these factors, the chromophore or fluorophore conjugated to the substrate can influence its hydrolysis rate and specificity, as well as the permeability of the substrate and its hydrolysis product.

Molecular Probes prepares a variety of protease substrates, including selective protease substrates for caspase-3 and caspase-8 — enzymes that are activated during apoptosis (Section 15.5) — HIV protease and renin. Our patented EnzChek and DQ protease substrates include:

- The green-fluorescent and red-fluorescent EnzChek Protease Assay Kits (E-6638, E-6639), which use a heavily BODIPY dye–labeled DQ casein derivative for the assay of a wide variety of proteases (Table 10.4, Figure 10.47). These products are also available in a RediPlate 96 and RediPlate 384 format for the convenient, high-throughput screening of protease inhibitors in either 96-well or 384-well microplates (R-22130, R-22132, R-22131, R-22133).
- The EnzChek Polarization Assay Kit for Proteases (E-6658), which uses a green-fluorescent BODIPY FL casein conjugate with an optimal degree of labeling for fluorescence polarization-based general protease assays (see "Fluorescence Polarization (FP)" in Section 1.4).
- The EnzChek Gelatinase/Collagenase Assay Kit (E-12055) and EnzChek Elastase Assay Kit (E-12056), which use DQ gelatin or DQ elastin as substrates, provide the speed, high sensitivity and convenience required for measuring gelatinase (collagenase) or elastase activity and for screening of protease inhibitors in a high-throughput format.
- DQ collagen (D-12052, D-12060), DQ BSA (D-12050, D-12051) and DQ ovalbumin (D-12053), which are useful for the general screening of protease activity and matrix metalloproteinase (MMP) inhibitors and for the study of antigen processing.

Additionally, we provide some antibodies to matrix metalloproteinases and fluorescent protease inhibitors, which are described below.

Peptidase Substrates

The carboxy terminus of single amino acids and short peptides can be conjugated to certain amine-containing fluorophores to create fluorogenic peptidase substrates. The dyes used to make these substrates are fluorescent at physiological pH; however, when the dyes are coupled in an

amide linkage to peptides, their absorption maxima are usually shortened significantly. The resulting substrates are sometimes fluorescent but with relatively short-wavelength emission spectra. In an extreme case such as that of rhodamine 110–based substrates, detectable long-wavelength absorbance and fluorescence are completely eliminated by amide formation. Peptidase activity releases the fluorophore, restoring its free-dye fluorescence.

UV Light–Excitable Substrates Based n 7-Aminoc umarins

7-Amino-4-methylcoumarin (AMC, A-191; Section 10.1) is a blue-fluorescent dye (Figure 10.3) whose peptide amides are used extensively as substrates for detecting enzymatic activity in cells, homogenates and solutions. The CBZ-L-phenylalanyl-L-arginine amide of AMC (A-6521) is a substrate for a variety of serine proteases, including cathepsins, kallikrein and plasmin.[3–12] AMC and 7-amino-4-trifluoromethylcoumarin (AFC) — a dye with somewhat longer-wavelength spectra than AMC (excitation/emission maxima of ~380/500 nm) at pH 7 — are released from the caspase-3, caspase-7 and caspase-8 substrates listed in Table 15.5. These caspases are activated during early stages of apoptosis (Section 15.5). The Z-DEVD-AMC substrate is also a component of the EnzChek Caspase-3 Assay Kit #1 (E-13183, see below).

7-Amino-4-chloromethylcoumarin (CMAC, C-2110; Section 10.1) is a mildly thiol-reactive analog of AMC; CMAC-based substrates yield fluorescent peptidase products with improved retention in live cells. The fluorogenic *t*-BOC-Leu-Met-CMAC substrate [13,14] (A-6520) has been used to measure calpain activity in hepatocytes following the addition of extracellular ATP.[15,16]

Visible Light–Excitable Substrates Based on Rhodamine 110

Rhodamine 110 (R110, R-6479; Section 10.1) is a visible light–excitable dye with stronger absorbance than AMC; R110-based substrates usually comprise two identical amino acids or peptides attached to a single fluorophore.[17] Molecular Probes' bisamide derivatives of rhodamine 110 are sensitive and selective substrates for assaying protease activity in solution or inside live cells. Originally developed by Walter Mangel and colleagues, these substrates comprise an amino acid or peptide covalently linked to each of R110's amino groups, thereby suppressing both its visible absorption and fluorescence.[18,19] Upon enzymatic cleavage, the nonfluorescent bisamide substrate is converted in a two-step process first to the fluorescent monoamide and then to the even more fluorescent R110 (Figure 10.4, Figure 10.44). The fluorescence intensities of the monoamide and of R110 are constant from pH 3–9. Both of these hydrolysis products exhibit spectral properties similar to those of fluorescein, with peak excitation and emission wavelengths of 496 nm and 520 nm, respectively, making them compatible with flow cytometers [20] and other instrumentation based on the argon-ion laser. Substrates based on R110 may also be useful for sensitive absorptimetric assays because the R110 dye has intense visible absorption ($\epsilon_{496\ nm}$ ~80,000 $cm^{-1}M^{-1}$ in pH 6 solution).

Molecular Probes prepares a variety of substrates based on the rhodamine 110 fluorophore (Table 10.2). Bis-(CBZ-Arg)-R110 (BZAR, R-6501) is a general substrate for serine proteases that has proven to be 50- to 300-fold more sensitive than the analogous AMC-based substrate.[18,19,21] This enhanced sensitivity can be attributed both to the greater fluorescence of the enzymatic product and to the enhanced reactivity of the cleavage site. In addition, BZAR inhibits guanidinobenzoatase activity in tumor cells.[21,22] The tripeptide derivative bis-(CBZ-Ile-Pro-Arg)-R110 (BZiPAR, R-6505) allows direct and continuous monitoring of enzyme turnover, making it useful for determining individual kinetic constants of fast-acting, irreversible trypsin inhibitors.[23] BZiPAR has been shown to enter intact cells where it is cleaved by lysosomal proteases.[24] Simultaneous measurement of enzymatic activity with BZiPAR and Ca^{2+} transients with fura-2 (F-1201, F-1221; Section 20.2) has been reported.[25] Bis-(CBZ-Phe-Arg)-R110 (R-6502) has been employed for flow cytometric analysis of the cysteine proteases cathepsin B and L in human monocytes and rat macrophages.[26–28]

Bis-(CBZ-Ala-Ala)-R110 amide [29] (R-6504) is a long-wavelength calpain substrate. Bis-(CBZ-Ala-Ala-Ala-Ala)-R110 (R-6506), an elastase substrate, has been used in a novel DNA detection assay.[30] Bis-(CBZ-Ala-Arg)-R110 (R-6508) is a fluorogenic substrate for both elastase and trypsin.[18,27] The bis-(tosyl-Gly-Pro-Arg) amide of rhodamine 110 (R-22124) is a selective substrate for thrombin.[31] Turnover of this substrate by thrombin on a membrane in the presence of thromboplastin produces both color and fluorescence that has been reported to model coagulation and blood clot formation. We also offer the human renin substrate 1 (R-2931, see below) for measuring the activity of this important blood-pressure-regulating enzyme.

EnzChek Caspase-3 Assay Kits and Caspase-3 and Caspase-8 Substrates

Members of the caspase (CED-3/ICE) family of proteases have been identified as crucial mediators of the complex biochemical events associated with apoptosis.[32–34] Caspase-3 (CPP32/apopain) is a key effector in the apoptotic pathway, amplifying the signal from initiator caspases (such as caspase-8) and signifying full commitment to cellular disassembly. The active caspase-3 protease recognizes the amino acid sequence Asp-Glu-Val-Asp (DEVD) and has been shown to cleave other caspases in the enzyme cascade, as well as poly(ADP-ribose) polymerase (PARP), DNA-dependent protein kinase, protein kinase Cδ and actin.[34,35]

EnzChek Caspase-3 Assay Kits #1 and #2

Molecular Probes' EnzChek Caspase-3 Assay Kits #1 and #2 (E-13183, E-13184), which contain AMC- and R110-labeled peptidase substrates, respectively, permit the detection of apoptosis by assaying for increases in caspase-3 and caspase-3–like protease activities (Figure 10.45, Figure 15.82). The activation of caspase-3 (CPP32/apopain), which has a substrate selectivity for the amino acid sequence Asp-Glu-Val-Asp (DEVD) and cleaves a number of different proteins, including poly(ADP-ribose) polymerase (PARP), DNA-dependent protein kinase, protein kinase Cδ and actin, is important for the initiation of apoptosis.[34,35] Both kits can be used to continuously measure the activity of caspase-3 and closely related proteases in cell extracts and purified enzyme preparations using a fluorescence microplate reader or fluorometer. AMC-based DEVD substrates, which yield blue fluorescence upon proteolytic cleavage, are widely used to monitor caspase-3 activity.[35,36] The longer-wavelength spectra and higher extinction coefficient of the green-fluorescent products of the R110-based substrate in Kit #2 (E-13184) should provide even greater sensitivity.[37,38] Each kit contains:

- The Z-DEVD-AMC substrate (in Kit #1, E-13183) *or* Z-DEVD-R110 substrate (in Kit #2, E-13184)
- A concentrated cell-lysis buffer
- A concentrated reaction buffer
- Ac-DEVD-CHO inhibitor, for confirming that the observed fluorescence signal is due to the activity of caspase-3–like proteases [35]
- AMC in Kit #1 E-13183 *or* R110 in Kit #2 (E-13184) as a reference standard
- A detailed protocol

Each kit provides a sufficient amount of each material for performing ~500 assays using a volume of 100 µL per assay. For information about additional kits and reagents for studying apoptosis, see Section 15.5.

RediPlate 96 EnzChek Caspase-3 Assay Kit

Our EnzChek Caspase-3 Assay Kit #2 is also available as a convenient RediPlate 96 EnzChek Caspase-3 Assay Kit (R-35100), which includes one 96-well microplate, contained in a resealable foil packet to ensure the integrity of the fluorogenic components, plus all necessary buffers and reagents for performing the assay. The enzyme sample to be assayed is added to the microplate in a suitable buffer, along with any compounds to be tested. Then, after incubation, the resultant

fluorescence is quantitated on a fluorescence-based microplate reader equipped with filters appropriate for the green-fluorescent R110, with excitation/emission maxima of 496/520 nm. The microplate consists of twelve removable strips, each with eight wells, allowing researchers to perform only as many assay as required for the experiment (Figure 8.54). Eleven of the strips (88 wells) are preloaded with the Z-DEVD-R110 substrate. The remaining strip, marked with blackened tabs, contains a dilution series of free R110 that may be used as a fluorescence reference standard. The reversible aldehyde inhibitor Ac-DEVD-CHO, supplied in a separate vial, can be used to confirm that the observed fluorescence signal in both induced and control cell populations is due to the activity of caspase-3–like proteases.[35] Table 10.3 summarizes our other RediPlate 96 and RediPlate 384 Assay Kits for protease activity (see below), phosphatase activity (Section 10.3) and dsDNA quantitation (Section 8.3). Significant discounts apply to purchases of multiple units of all of our RediPlate products.

Caspase-3 Substrates

The Z-DEVD-R110 substrate,[37,38] a component of our EnzChek Caspase-3 Assay Kit #2 (E-13184) and RediPlate 96 EnzChek Caspase-3 Assay Kit (R-35100), is also available separately in a 20 mg unit size for high-throughput screening applications (R-22120, Table 10.2). This nonfluorescent bisamide is first converted by caspase-3 (or a closely related protease) to the fluorescent monoamide and then to the even more fluorescent rhodamine 110 (excitation/emission maxima ~496/520 nm). The bis-L-aspartic acid amide of R110 (R-22122, Table 10.2) contains only the rhodamine 110 fluorophore flanked by aspartic acid residues. This compound does not appear to require any invasive techniques like osmotic shock to gain entrance into the cytoplasm, and may serve as a substrate for a variety of apoptosis related proteases, including caspases-3 and -7 [37] (Figure 15.85).

Caspase-8 Substrates

Caspase-8 plays a critical role in the early cascade of apoptosis, acting as an initiator of the caspase activation cascade. Activation of the enzyme itself is accomplished through direct interaction with the death domains of cell surface receptors for apoptosis inducing ligands.[39,40] The activated protease has been shown to be involved in a pathway that mediates the release of cytochrome *c* from the mitochondria [41] and is also known to activate downstream caspases, such as caspase-3.[42] Three fluorogenic substrates containing the caspase-8 recognition sequence Ile-Glu-Thr-Asp (IETD) are available (Table 15.5): (A-22127; blue fluorescent after cleavage), (A-22128; blue-green–fluorescent after cleavage) and Z-IETD-R110 (R-22125, R-22126; green fluorescent after cleavage).

Substrates for HIV Protease and Renin

Alternative strategies have been employed to create substrates specifically for some endopeptidases. Our HIV protease and renin substrates (H-2930, R-2931) utilize fluorescence resonance energy transfer (FRET, see Section 1.3) to generate a spectroscopic response to protease cleavage. In this type of substrate, both an acceptor molecule and a fluorescent molecule are attached to the peptide or protein. The acceptor molecule is carefully chosen so that its absorbance overlaps with the fluorophore's excited-state fluorescence (Figure 10.46), thus ensuring that the fluorescence is quenched through resonance energy transfer.[43] Enzyme hydrolysis of the substrate results in spatial separation of the fluorophore and the acceptor molecule, thereby restoring the fluorophore's fluorescence (Figure 10.9). See Section 9.5 for a discussion of our reagents for synthesizing labeled peptides and peptidase substrates, including our QSY series of nonfluorescent dyes (Figure 1.73), which have broad visible or near infrared absorption spectra and serve as almost universal quenchers of most fluorescent donors that emit in the visible, with unusually high efficiency (Table 1.10).

Substrate f r Detecting HIV Protease Activity

HIV protease substrate 1 (H-2930) is a peptide that includes the HIV protease cleavage site, along with two covalently modified amino acid residues — one that has been linked to EDANS and the other to dabcyl.[44–46] Proteolytic cleavage releases a fragment containing only the EDANS fluorophore, thus liberating it from the quenching effect of the nearby dabcyl chromophore (Figure 10.9). HIV protease activity can be measured by exciting the sample at ~340 nm and measuring the resulting fluorescence at 490 nm. HIV protease substrate 1 has been used to analyze the effects of solvent composition, incubation time and enzyme concentration on HIV-1 protease activity [47] and to investigate a newly designed inhibitor of the enzyme.[48] HIV protease substrate 1 has also been employed to follow the inhibition of HIV-1 protease activity after the enzyme's two cysteine residues are reversibly modified by nitric oxide.[49] One milligram of HIV protease substrate 1 is sufficient for approximately 120 enzyme assays using 2 mL assay volumes and standard fluorescence cuvettes or ~1600 assays using 150 µL assay volumes and microcuvettes.

Human Renin Substrate

Assaying renin activity with human renin substrate 1 (R-2931) is analogous to assaying HIV protease activity with the HIV protease substrate described above. Renin, an aspartic protease, plays an important role in blood-pressure regulation and is therefore a target for anti-hypertension therapeutics. Using renin substrate 1, researchers have discovered a stable, partially active conformational variant of recombinant human prorenin.[50] This substrate has also been used to investigate the kinetics and pH stability of recombinant human renin.[51] A fluorogenic substrate similar to our renin substrate 1 was used to develop a microplate-based assay for screening renin inhibitors.[52] One milligram of the renin substrate 1 is sufficient for approximately 100 enzyme assays using 2 mL volumes and standard fluorescence cuvettes or ~1400 assays using 150 µL assay volumes and microcuvettes. Molecular Probes also has a fluorogenic substrate for thrombin (rhodamine 110, bis-(tosyl-Gly-Pro-Arg) amide, R-22124; see above) that is useful for measuring the activity of this enzyme, which is important for coagulation and blood clot formation.[31]

EnzChek Protease Assay Kits and Fluorescein Casein

Often it is necessary to have fluorogenic substrates for the assay of purified enzymes with unknown specificity or for which there are no known useful substrates. Assay for contamination of biological preparations by unknown proteases requires substrates that can detect a variety of enzymes. Our patented method of relieving the fluorescence quenching of BODIPY dye–labeled biopolymers by enzymatic hydrolysis (Figure 10.47) has been used in several of the general or selective protease assay kits and DQ reagents described in this section. We have also described the method's use in an assay for dextranase,[53] and others have applied it to the assay of *O*-sialoglycoprotein endopeptidase [54] and enzymes that process vesicular stomatitis virus (VSV).[55] The DQ and EnzChek protease assay reagents may have significant potential for detecting matrix metalloproteinase (MMP) activity in living tissues by simple incubation of the tissue with the protein-based fluorogenic substrates.[2] Fluorescent products have been shown to accumulate on the cell's surface where proteases are active, including in living human breast cancer cells.[56] These quenched protease substrates are particularly useful for following cell migration through matrices [57–59] (Figure 10.48).

The EnzChek Protease Assay Kits provide exceptionally fast, simple and direct fluorescence assays for detecting metallo-, serine, acid and thiol proteases. Our two EnzChek Protease Assay Kits (E-6638, E-6639) measure the increase in fluorescence intensity that results from protease hydrolysis of a heavily labeled casein derivative, whereas our EnzChek Polarization Assay Kit for Proteases (E-6658) monitors fluorescence polarization changes that occur during protease hydrolysis of a lightly labeled fluorescent casein derivative. Although the detection principles of these protease assays are quite different, no separation steps are required for either, and both assays are rapid, sensitive and versatile.

EnzChek Pr tease Assay Kits for Fluorescence Intensity Measurements

Our patented EnzChek Protease Assay Kits contain a casein derivative that is heavily labeled with either the green-fluorescent BODIPY FL or red-fluorescent BODIPY TR-X dye, resulting in almost total quenching of the conjugate's fluorescence; they typically exhibit <3% of the fluorescence of the corresponding free dyes.[60–63] Protease-catalyzed hydrolysis relieves this quenching, yielding brightly fluorescent BODIPY FL dye– or BODIPY TR-X dye–labeled peptides [61,62] (Figure 10.47). The increase in fluorescence, which can be measured with a spectrofluorometer, minifluorometer or fluorescence microplate reader, is directly proportional to protease activity.

In contrast to the conventional fluorescein thiocarbamoyl (FTC)–casein protease assay, these EnzChek assays do not involve any separation steps and, consequently, can be used to continuously measure the kinetics of a variety of exopeptidases and endopeptidases over a wide pH range. They can also be used to measure the total substrate turnover at a fixed time following addition of the enzyme. We have found that these protease assays are over 100-times more sensitive and much easier to perform than the labor-intensive FTC–casein assay. Detection limits for fluorescence intensity measurements with these kits are given in Table 10.4.

Hydrolysis of the fluorogenic substrates by proteases provides a sensitive assay of cell proliferation and a means of detecting the sterility of a sample. In addition to their utility for detecting protease contamination in culture medium and other experimental samples, BODIPY FL casein and BODIPY TR-X casein have significant potential as general, nontoxic, pH-insensitive markers for phagocytic cells in culture (Section 16.1). We have shown that uptake of these quenched conjugates by neutrophils is accompanied by hydrolysis of the labeled proteins by intracellular proteases and generation of fluorescent products that are well retained in cells. This phagocytosis assay is readily performed in a fluorescence-based microplate reader or a flow cytometer; localization of the fluorescent products can be determined by fluorescence microscopy. The same substrates can readily detect secretion of proteases from live cells.

BODIPY FL casein and BODIPY TR-X casein can be used interchangeably, depending on whether green or red fluorescence is desired. The peptide hydrolysis products of BODIPY FL casein exhibit green fluorescence that is optimally excited by the argon-ion laser, permitting flow sorting of the cells. The red-fluorescent BODIPY TR-X–labeled peptides, with excitation and emission spectra similar to those of the Texas Red fluorophore, should be useful for multilabeling experiments or measurements in the presence of green autofluorescence. Each EnzChek Protease Assay Kit includes:

- BODIPY FL casein in Kit E-6638 *or* BODIPY TR-X casein in Kit E-6639
- A 20x digestion buffer
- A detailed protocol

A sufficient amount of each reagent is supplied for ~100 assays using 2 mL assay volumes and standard fluorescence cuvettes or ~1000 assays using 200 µL assay volumes and 96-well microplates.

Our EnzChek Protease Assay Kits are also available as convenient RediPlate 96 and RediPlate 384 EnzChek Protease Assay Kits (R-22130; R-22132). Each RediPlate 96 EnzChek Protease Assay Kit includes one 96-well microplate, with all of the necessary reagents predispensed into the wells, where 88 wells (11 lanes) are intended for assays and 8 wells (1 lane) include a dilution series of an appropriate reference standard for generation of standard curves. The enzyme sample to be assayed is added to the microplate in a suitable buffer, along with any compounds to be tested. Then, after incubation, the resultant fluorescence is quantitated on a fluorescence-based microplate reader equipped with filters appropriate for the green- or red-fluorescent dye. Each RediPlate 96 microplate has removable lanes that allow researchers to perform only as many assays as required for the experiment (Figure 8.54). Resealable packaging ensures plate and well integrity between experiments.

The RediPlate 384 EnzChek Protease Assay Kits (R-22131; R-22133) include a single 384-well plate for high-throughput screening of protease activity. These standard 384-well microplates include 368 wells (23 lanes) for activity measurements and 16 wells (1 lane) containing a dilution series of an appropriate reference dye for generation of standard curves.

Table 10.3 summarizes our other RediPlate 96 and RediPlate 384 Assay Kits for caspase-3 activity (see above), phosphatase activity (Section 10.3) and dsDNA quantitation (Section 8.3). Significant discounts apply to purchases of multiple units of all of our RediPlate products.

EnzChek Protease Assay Kit for Fluorescence Polarization Measurements

When a fluorescent molecule tethered to a protein is excited by polarized fluorescent light, the polarization of fluorescence emission is dependent on the rate of molecular tumbling. Upon proteolytic cleavage of the fluorescently labeled protein, the resultant smaller peptides tumble faster, and the emitted light is depolarized relative to the light measured from the intact conjugate. The EnzChek Polarization Assay Kit for Proteases [64] (E-6658) contains green-fluorescent BODIPY FL casein with an optimal degree of labeling for fluorescence polarization–based protease assays.[65,66] Fluorescence polarization technology is more sensitive than many nonradioactive protease assays and allows measurements to be taken in real time, permitting the collection of kinetics data (see "Fluorescence Polarization (FP)" in Section 1.4). Our BODIPY FL dye has an adequate fluorescence lifetime and pH-insensitive fluorescence — two prerequisites for successful measurement of protease activity by fluorescence polarization. The EnzChek Polarization Assay Kit for Proteases contains:

- BODIPY FL casein
- A 5x digestion buffer
- A detailed protocol

A sufficient amount of each reagent is supplied for ~100 assays using 2 mL assay volumes and standard fluorescence cuvettes or ~1000 assays using 200 µL assay volumes and 96-well microplates. With the advent of high-capacity automated instrumentation, this kit provides an important tool for high-throughput screening of proteases and their inhibitors in research laboratories.

Fluorescein Casein

We also offer fluorescein casein (C-2990) for assaying protease activity. In this assay, unhydrolyzed fluorescein casein must be precipitated with trichloroacetic acid, separated by centrifugation, transferred for measurement and then pH-adjusted for fluorescein signal enhancement.[67–71] Fluorescein casein may be useful for a continuous assay if monitored by fluorescence polarization.[66] Fluorescein casein is rapidly degraded by *Bacteroides gingivalis* but only slowly by streptococci.[72]

EnzChek Gelatinase/Collagenase Assay Kit

Collagen is a major component of the extracellular matrix, which not only serves as scaffolding to stabilize tissue structure, but also influences the development, migration, proliferation and metabolism of cells that contact it. Gelatinases and collagenases — matrix metalloproteinases (MMPs) that digest collagen or gelatin (denatured collagen) — are increasingly important to our understanding of both normal development and carcinogenesis.[73] For example, gelatinase A (20,000-dalton MMP-2) is primarily responsible for degrading the helical domains of type IV collagen, the principal collagen of basement membranes.[74] Thus, gelatinase A likely plays a major role in the turnover of basement membrane during fetal tissue development, wound healing, angiogenesis and tumor invasion.

The EnzChek Gelatinase/Collagenase Assay Kit (E-12055) provides the speed, high sensitivity and convenience required for measuring gelatinase or collagenase activity or for screening inhibitors [75] in a high-throughput format. This kit contains:

- DQ gelatin, a gelatin conjugate so heavily labeled with fluorescein that its fluorescence is quenched; this substrate is also available separately (D-12054)
- 1,10-Phenanthroline, a general metalloproteinase inhibitor
- Type IV collagenase from *Clostridium histolyticum* for use as a positive control
- A 10× reaction buffer
- A detailed protocol

DQ gelatin, the highly quenched gelatinase/collagenase substrate provided, is efficiently digested by most, if not all, gelatinases and collagenases, releasing brightly fluorescent peptides (Figure 10.47). The increase in fluorescence upon digestion is proportional to proteolytic activity and can be monitored with a fluorescence microplate reader, minifluorometer or spectrofluorometer. Depending on the substrate concentration used in each reaction, a sufficient amount of each reagent is supplied for 250–1000 assays using 200 µL assay volumes and 96-well microplates. Gelatinase activity in living oligodendrocytes has been localized without the use of fixatives in an *in situ* assay.[76]

Using 100 µg/mL DQ gelatin and a two-hour incubation period, it is possible to detect down to 2×10^{-3} U/mL (7 ng protein/mL) of *C. histolyticum* collagenase, where one unit is defined as the amount of enzyme required to liberate 1 µmole L-leucine equivalents from collagen in 5 hours at 37°C, pH 7.5. Longer incubation times increase the sensitivity, whereas higher enzyme concentrations decrease the incubation times. Using human gelatinase A (not provided), 100 µg/mL DQ gelatin and a 24-hour incubation period, we have detected concentrations of gelatinase as low as 3×10^{-4} U/mL, where one unit is defined as the amount of enzyme that can hydrolyze 1 mg of type IV collagen in one hour at 37°C, pH 7.5.

EnzChek Elastase Assay Kit

Molecular Probes' EnzChek Elastase Assay Kit (E-12056) provides the speed, high sensitivity and convenience required for measuring elastase activity or for screening inhibitors in a high-throughput format. This kit contains DQ elastin — soluble bovine neck ligament elastin that has been labeled with our BODIPY FL dye such that the conjugate's fluorescence is quenched.[63] Upon digestion by elastase or other proteases, the fluorescence is revealed (Figure 10.47). The resulting increase in fluorescence can be monitored with a fluorescence microplate reader, minifluorometer or spectrofluorometer. Digestion products from the DQ elastin substrate have absorption maxima at ~505 nm and fluorescence emission maxima at ~515 nm. Because the assay is continuous, kinetics data can be obtained easily. Furthermore, because fluorescence of the BODIPY FL dye is pH insensitive between pH 3 and 9, the assay can be performed under a variety of buffer conditions. Please note that DQ elastin is also digested by proteases other than elastase. Each EnzChek Elastase Assay Kit (A-10256) contains:

- The DQ elastin substrate
- A 10× reaction buffer
- Elastase from pig pancreas for use as a positive control
- *N*-Methoxysuccinyl-Ala-Ala-Pro-Val-chloromethyl ketone, a selective elastase inhibitor
- A detailed protocol

Each kit provides a sufficient amount of each reagent for approximately 600 assays, using a fluorescence microplate reader and reaction volumes of 200 µL. The *N*-methoxysuccinyl-Ala-Ala-Pro-Val-chloromethyl ketone inhibitor can be used to confirm the identity of the protease responsible for substrate digestion or, alternatively, as a control inhibitor for use when screening for elastase inhibitors.

DQ Substrates

DQ C llagens

Our DQ collagens, type I (D-12060) and type IV (D-12052) are complementary reagents to the DQ gelatin provided in the EnzChek Gelatinase/Collagenase Assay Kit. Like DQ gelatin, these highly quenched substrates are heavily labeled with fluorescein and release fluorescent peptides when enzymatically cleaved. DQ collagen, type I should be useful in assays detecting matrix metalloproteases (MMP-1) activity.[77] DQ collagen, type IV may prove particularly useful in the development of assays for gelatinase A (MMP-2) and gelatinase A inhibitors, as well as for other gelatinases and collagenases that specifically degrade type IV collagen. DQ collagens may be used with the EnzChek Gelatinase/Collagenase Assay Kit, and a sample protocol is included. Because of its more complex structure, DQ collagens generally require either greater amounts of the enzyme or longer incubation periods than does DQ gelatin. Please note that both the DQ collagens and DQ gelatin can be digested by proteases other than gelatinases and collagenases. The fluorescence generated by hydrolysis of DQ collagen (D-12052) by cellular collagenase has been used to visualize the migratory pathway followed by tumor cells during invasion of a gelatin matrix and to image proteolysis by living breast cancer cells.[56,57]

In addition to the DQ substrates, we have prepared gelatin and collagen conjugates that have been labeled to maximize probe fluorescence and minimize dye quenching. We offer two green-fluorescent conjugates of gelatin, one in which gelatin is coupled to our photostable Oregon Green 488 dye (G-13186, Section 15.6) and the other to fluorescein (G-13187, Section 15.6).

DQ BSA

DQ Green BSA (D-12050, excitation/emission maxima 500/506 nm) and DQ Red BSA (D-12051, excitation/emission maxima 589/617 nm) are bovine serum albumin (BSA) conjugates that have been labeled to such a high degree that the BODIPY dyes used to label them are strongly self-quenched.[63] Proteolysis of the DQ BSA can easily be monitored as proteolytic fragments containing the fluorophores are released from the larger conjugate and become brightly fluorescent [78] (Figure 10.47). An unlabeled neutrophil making its way through a gelatin matrix containing a DQ Green BSA prototype, as well as dihydrotetramethylrosamine, a nonfluorescent probe that fluoresces bright orange upon oxidation, leaves behind a fluorescent trail [59] (Figure 10.48). The alternating green- and orange-fluorescent bands dramatically demonstrate that the proteolytic and oxidative activities of the migrating neutrophil are oscillatory and are 180° out of phase with each other. Intracellular processing of a similar BODIPY BSA conjugate by J774 macrophages can be completely inhibited by protease inhibitors.[78] DQ Green BSA has been embedded in a gelatin matrix and used to image both extracellular and intracellular proteolysis in living cells. DQ BSA can be complexed with our rabbit IgG fraction anti-BSA (A-11133, Section 7.5) to form an immune complex that is internalized through the Fc receptor and processed in the phagovacuole to highly fluorescent peptides (Section 16.1). DQ Green BSA has been used for imaging proteolysis in living breast cancer cells.[56]

DQ Ovalbumin

DQ ovalbumin [79,80] (D-12053) is a self-quenched ovalbumin conjugate designed specifically for the study of antigen processing. Ovalbumin is efficiently processed through mannose receptor–mediated endocytosis by antigen-presenting cells and is widely used for studying antigen processing.[81–83] DQ ovalbumin is labeled with our pH-insensitive, green-fluorescent BODIPY FL dye such that the fluorescence is almost completely quenched.[63] Upon endocytosis and proteolysis, highly fluorescent peptides are released. DQ ovalbumin appears to be an excellent indicator of macrophage-mediated antigen processing in flow cytometer– and microscope-based assays.

Matrix Metall pr teinases

The matrix metalloproteinases (MMPs) constitute a family of zinc-dependent endopeptidases that function within the extracellular matrix. These enzymes are responsible for the breakdown of connective tissues and are important in bone remodelling, the menstrual cycle and repair of tissue damage. While the exact contribution of MMPs to certain pathological processes is difficult to assess, MMPs appear to have a key role in the development of arthritis and the invasion and metastatis of cancer.[84,85]

MMPs tend to have multiple substrates, with most family members having the ability to degrade several different types of collagen along with elastin, gelatin and fibronectin.[86] Most MMPs contain three major domains: a regulatory domain (which must be removed before the enzyme can be active), a catalytic domain and a hemopexin domain. The hemopexin domain aids in enzyme binding to certain substrates, although it is not necessary for the catalytic function of the enzyme.[87]

To assist researchers in the study of these important enzymes, Molecular Probes offers high activity rabbit antibodies against MMP-1, MMP-2, MMP-3 and MMP-9 (Table 7.19). In each case the antibody is directed at the stretch of amino acids that form the small hinge region between the catalytic and hemopexin domain (Figure 7.83). The hinge region is highly variable among MMPs,[86] therefore antibodies raised against this peptide sequence have very little crossreactivity with other MMPs. All of these antibodies recognize both the pro (inactive) and active forms of their respective MMP target, and are suitable for Western blotting, immunoprecipitation and immunohistochemistry applications.

Alternative Methods for Detecting Protease Activity

Peptidases typically liberate a free amine for each hydrolysis step. Thus, fluorogenic amine detection reagents such as fluorescamine (F-2332, FluoroPure Grade F-20261; Section 1.8) and *o*-phthaldialdehyde (P-2331, Section 1.8) have been used to detect the rate of amine production by peptidases.[88–91]

Peptidases that liberate single free amino acids for which specific oxidases exist can be analyzed by coupling the hydrolytic reaction to oxidation of our Amplex Red reagent (Section 10.5) to the red-fluorescent dye resorufin (Figure 10.5). For example, glutamic acid production can be monitored using glutamate oxidase [92] and D-amino acid liberation monitored using a D-amino acid oxidase.

Protease assays conducted in highly autofluorescent or strongly light-scattering solutions (such as crude cell and tissue extracts) often can be improved by extracting the fluorescent hydrolysis product from the assay mixture with an organic solvent such as toluene, chloroform or ethyl acetate.[93] Most unhydrolyzed peptidase substrates will remain in the aqueous layer.

Endopeptidase substrates that are singly labeled at the amine terminus with a fluorophore usually do not undergo a fluorescence change upon hydrolysis of internal peptide bonds; however, fluorescence (or absorbance) of the fluorophore that remains attached to the cleaved peptide can be used to detect the hydrolysis product following separation by TLC, HPLC or capillary electrophoresis.

Flu rescent Protease Inhibitors

Alexa Fluor 488 Soybean Trypsin Inhibitor

Trypsin inhibitor from soybean (SBTI) is a 21,000-dalton protein that inhibits the catalytic activity of serine proteases.[94,95] SBTI binds to acrosin, an acrosomal serine protease that is associated with

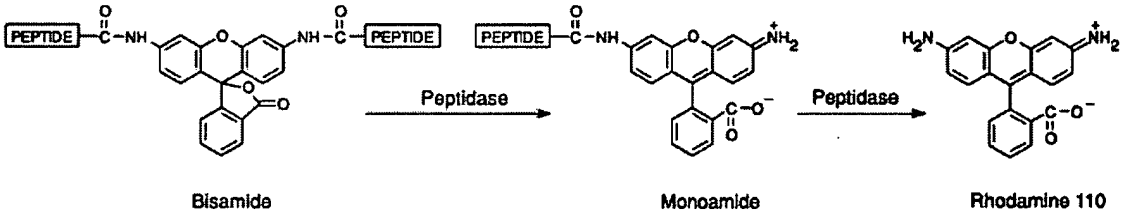
binding of spermatozoa and penetration of the zona pellucida,[96,97] and SBTI binding patterns in non-fixed human sperm have demonstrated its usefulness for detecting acrosome-reacted sperm.[98] In particular, an Alexa Fluor 488 conjugate of the protein has been used to measure the acrosomal status of macaque sperm and to determine the localization of acrosin during the reaction.[99] Molecular Probes provides a fluorescent SBTI conjugate with one of our best fluorophores, the Alexa Fluor 488 dye (T-23011). The Alexa Fluor 488 dye (Section 1.3) has spectral characteristics similar to fluorescein (excitation at 495 nm and emission at 519 nm) but produces conjugates that are brighter and more photostable. Furthermore, the fluorescence of Alexa Fluor 488 conjugates is insensitive to pH from 4 to 10 (Figure 1.12).

Pepstatin A (P-6542) is an inhibitor of carboxyl (acid) proteases that contain aspartate residues at their active sites, including cathepsin D, pepsin and renin.[100–102] Molecular Probes has prepared the green-fluorescent BODIPY FL pepstatin A analog (P-12271), which we have shown binds to cathepsin D in live cells [103] (Section 15.5).

1. Methods Enzymol 244, 1 (1994); **2.** J Histochem Cytochem 49, 1473 (2001); **3.** J Biol Chem 270, 558 (1995); **4.** Arch Biochem Biophys 314, 171 (1994); **5.** FEBS Lett 341, 197 (1994); **6.** J Biochem (Tokyo) 113, 441 (1993); **7.** FEBS Lett 257, 388 (1989); **8.** Arch Biochem Biophys 259, 131 (1987); **9.** Biochem J 201, 367 (1982); **10.** Biochem J 193, 187 (1981); **11.** Methods Enzymol 80 Pt C, 341 (1981); **12.** J Biochem (Tokyo) 82, 1495 (1977); **13.** J Biol Chem 275, 2390 (2000); **14.** J Biol Chem 275, 9452 (2000); **15.** J Biol Chem 268, 23593 (1993); **16.** J Biol Chem 274, 787 (1999); **17.** Symmetric rhodamine 110–based substrates are licensed to Molecular Probes under US 4,557,862 and US 4,640,893 and are sold for nonclinical, research and developmental purposes only; **18.** Biochem J 215, 253 (1983); **19.** Biochem J 209, 299 (1983); **20.** Cytometry 20, 334 (1995); **21.** Anticancer Res 8, 1179 (1988); **22.** J Enzyme Inhib 2, 209 (1988); **23.** Biochim Biophys Acta 788, 74 (1984); **24.** Photochem Photobiol 44, 461 (1986); **25.** Proc Natl Acad Sci U S A 97, 13126 (2000); **26.** Glia 7, 183 (1993); **27.** Biol Chem Hoppe Seyler 373, 547 (1992); **28.** Biol Chem Hoppe Seyler 373, 433 (1992); **29.** Neuron 20, 1123 (1998); **30.** Anal Chem 65, 2352 (1993); **31.** Biomed Instrum Technol 30, 245 (1996); **32.** Immunol Cell Biol 76, 1 (1998); **33.** Science 281, 1312 (1998); **34.** Trends Biochem Sci 22, 388 (1997); **35.** Nature 376, 37 (1995); **36.** J Neurosci Res 52, 334 (1998); **37.** Biochemistry 38, 13906 (1999); **38.** Bioorg Med Chem Lett 9, 3231 (1999); **39.** Cell Death Differ 6, 821 (1999); **40.** Science 281, 1305 (1998); **41.** J Biol Chem 274, 17484 (1999); **42.** Exp Cell Res 250, 203 (1999); **43.** J Biochem Biophys Methods 33, 135 (1996); **44.** Science 247, 954 (1990); **45.** Tetrahedron Lett 31, 6493 (1990); **46.** J Biol Chem 272, 15603 (1997); **47.** J Biol Chem 267, 20028 (1992); **48.** Science 249, 527 (1990); **49.** Biochemistry 38, 13407 (1999); **50.** J Protein Chem 9, 663 (1990); **51.** J Protein Chem 10, 553 (1991); **52.** Anal Biochem 210, 351 (1993); **53.** Anal Biochem 260, 257 (1998); **54.** Anal Biochem 259, 8 (1998); **55.** J Virol Methods 70, 45 (1998); **56.** Neoplasia 2, 496 (2000); **57.** FASEB J 15, 932 (2001); **58.** Exp Cell Res 260, 292 (2000); **59.** Biophys J 74, 90 (1998); **60.** Anal Biochem 279, 170 (2000); **61.** Anal Biochem 251, 144 (1997); **62.** Anal Biochem 254, 144 (1997); **63.** US 5,719,031; **64.** Anal Biochem 243, 1 (1996); **65.** J Biomol Screen 1, 33 (1996); **66.** Biotechniques 17, 585 (1994); **67.** Cytometry 15, 213 (1994); **68.** Anal Biochem 197, 347 (1991); **69.** Anal Biochem 191, 133 (1990); **70.** Anal Biochem 143, 30 (1984); **71.** Anal Biochem 121, 290 (1982); **72.** FEMS Microbiol Lett 55, 257 (1990); **73.** Chem Biol 3, 895 (1996); **74.** J Biol Chem 270, 5872 (1995); **75.** J Biol Chem 276, 11347 (2001); **76.** J Neurosci 19, 8464 (1999); **77.** Lab Invest 78, 687 (1998); **78.** Eur J Cell Biol 75, 192 (1998); **79.** J Immunol 165, 49 (2000); **80.** Proc Natl Acad Sci U S A 96, 15056 (1999); **81.** Eur J Immunol 25, 1823 (1995); **82.** J Immunol 149, 2894 (1992); **83.** J Immunol 145, 417 (1990); **84.** Int J Oncol 12, 1343 (1998); **85.** Mol Med Today 4, 130 (1998); **86.** Matrix Metalloproteinases and TIMPs, Woessner JF 11 (2000); **87.** Matrix Biol 15, 511 (1997); **88.** Biochemistry 29, 6670 (1990); **89.** Anal Biochem 123, 41 (1982); **90.** Anal Biochem 87, 556 (1978); **91.** Biochem Biophys Res Commun 53, 75 (1973); **92.** Anal Biochem 284, 382 (2000); **93.** Anal Biochem 87, 257 (1978); **94.** J Biol Chem 241, 3955 (1966); **95.** Methods Enzymol 45, 700 (1976); **96.** Development 109, 41 (1990); **97.** Zygote 7, 143 (1999); **98.** Fertil Steril 62, 1044 (1994); **99.** Zygote 8, 127 (2000); **100.** J Biol Chem 267, 24725 (1992); **101.** Biochemistry 24, 3165 (1985); **102.** J Antibiot (Tokyo) 29, 97 (1976); **103.** J Biochem Biophys Methods 42, 137 (2000).

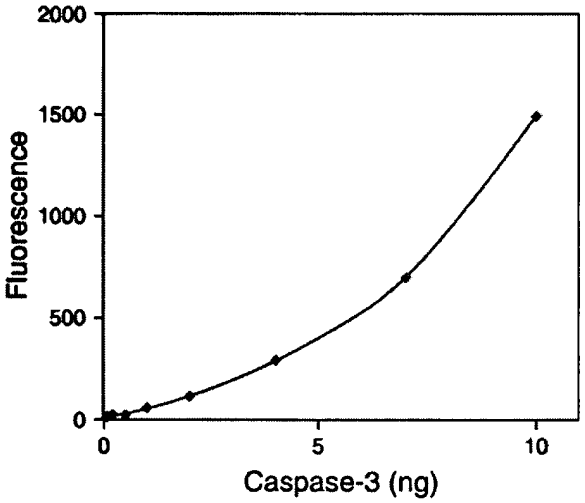
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Figure 10.44 Sequential peptidase cleavage of a rhodamine 110–based substrate. The nonfluorescent bisamide substrate is first converted to the fluorescent monoamide and then to the highly fluorescent rhodamine 110.



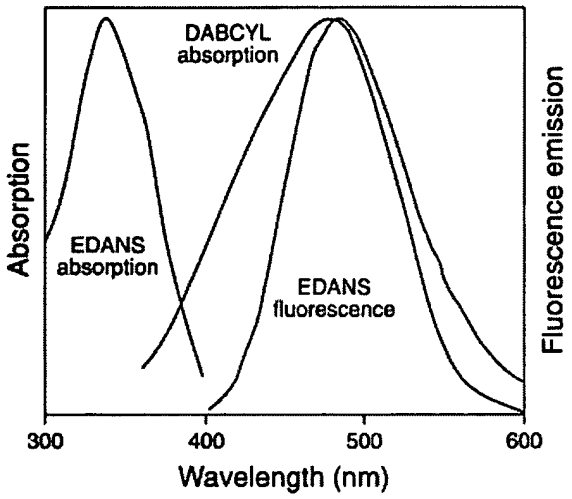
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Figure 10.45 Detection of caspase-3 activity using the EnzChek Caspase-3 Assay Kit #1 (E-13183). Increasing amounts of purified active human (recombinant) caspase-3 (PharMingen) were allowed to react with 100 µM Z-DEVD–AMC in 1x reaction buffer for ~45 minutes at room temperature. Fluorescence was measured in a fluorescence microplate reader using excitation at 360 ± 17.5 nm and emission detection at 465 ± 17.5 nm. Background fluorescence (386 arbitrary units), determined for a no-enzyme control, was subtracted from each value.



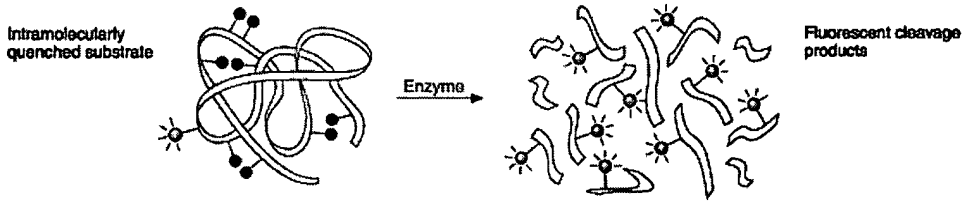
* * * * *

Figure 10.46 Spectral overlap between EDANS fluorescence and dabcyl absorption, which is required for efficient quenching of EDANS fluorescence by resonance energy transfer to the nonfluorescent dabcyl chromophore. Spectra are normalized to the same intensities.



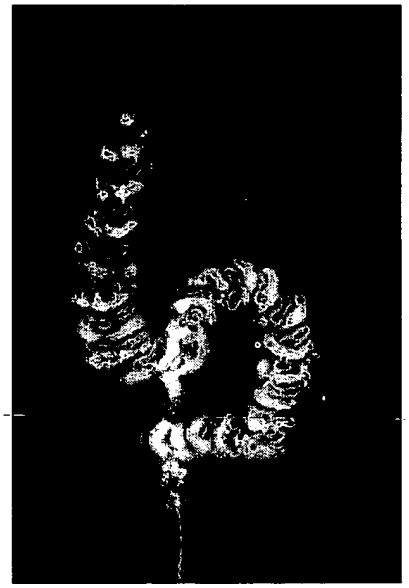
* * * * *

Figure 10.47 Principle of enzyme detection via the disruption of intramolecular self-quenching. Enzyme-catalyzed hydrolysis of the heavily labeled and almost totally quenched substrates provided in our EnzChek Protease Assay Kits (E-6638, E-6639), EnzChek Amylase Assay Kit (E-11954), EnzChek Gelatinase/Collagenase Assay Kit (E-12055), EnzChek Elastase Kit (E-12056), EnzChek Lysozyme Assay Kit (E-22013) — as well as the standalone quenched substrates DQ BSA (D-12050, D-12051), DQ collagen (D-12052, D-12060), DQ ovalbumin (D-12053) and DQ gelatin (D-12054) — relieves the intramolecular self-quenching, yielding brightly fluorescent reaction products.



* * * * *

Figure 10.48 Unlabeled neutrophils that have been allowed to migrate spontaneously in gelatin matrices containing a prototype of DQ Green BSA (D-12050) and dihydrotetramethylrosamine. Extracellular release of reactive oxygen metabolites oxidizes dihydrotetramethylrosamine to tetramethylrosamine, yielding orange fluorescence. Extracellular protease activity cleaves highly quenched DQ Green BSA, yielding green-fluorescent peptides. The micrograph illustrates an alternating pattern of orange and green fluorescence and shows that extracellular release of oxidants and proteolytic activity oscillate 180° out of phase during cell locomotion. Image contributed by Howard R. Petty, Wayne State University.



* * * * *

Table 10.2 — Rhodamine 110–based bis-peptide substrates

Cat #	Proteinase Substrate *	Enzymes
R-22122	$(Asp)_2$-R110	caspase-3 [1]
R-6504	$(CBZ\text{-}Ala\text{-}Ala)_2$-R110	elastase [2]
R-6506	$(CBZ\text{-}Ala\text{-}Ala\text{-}Ala\text{-}Ala)_2$-R110	elastase [3]
R-6508	$(CBZ\text{-}Ala\text{-}Arg)_2$-R110	trypsin [4]
R-6501	$(CBZ\text{-}Arg)_2$-R110	trypsin [4,5]
R-22120	$(CBZ\text{-}Asp\text{-}Glu\text{-}Val\text{-}Asp)_2$-R110	caspase-3 [1]
R-6505	$(CBZ\text{-}Ile\text{-}Pro\text{-}Arg)_2$-R110	trypsin [6,7]
R-6502	$(CBZ\text{-}Phe\text{-}Arg)_2$-R110	plasmin, cathepsin L [2,8,9]
R-22124	$(p\text{-tosyl-}Gly\text{-}Pro\text{-}Arg)_2$-R110	thrombin [10]
R-22123	$(SC\text{-}Ala\text{-}Ala\text{-}Pro\text{-}Phe)_2$-R110	chymotrypsin
* CBZ = benzyloxycarbonyl; R110 = rhodamine 110; SC = succinoyl; *p*-tosyl = *p*-toluenesulfonyl		

1. Biochemistry 38, 13906 (1999); **2.** Biol Chem Hoppe Seyler 373, 547 (1992); **3.** Anal Chem 65, 2352 (1993); **4.** Biochem J 215, 253 (1983); **5.** Biochem J 209, 299 (1983); **6.** Biochim Biophys Acta 788, 74 (1984); **7.** Photochem Photobiol 44, 461 (1986); **8.** Glia 7, 183 (1993); **9.** Biol Chem Hoppe Seyler 373, 433 (1992); **10.** Biomed Instrum Technol 30, 245 (1996).

* * * * *

Table 10.3 — RediPlate Assay Kits — One-step Fluorescent Microplate Assays

RediPlate Assay Kit	Cat. #		Assay Details
	96-well	384-well	
EnzChek Caspase-3 Assay Kit	(R-35100)	Inquire	A direct fluorescence-based assay for detecting caspase-3 and other DEVD-selective protease activities (e.g., caspase-7) in cell extracts and purified enzyme preparations. Caspase activity is indicated by increasing green fluorescence (excitation/emission maxima ~496/520 nm).
EnzChek Protease Assay Kit *green fluorescence*	(R-22130)	(R-22131)	A direct fluorescence-based assay for detecting metallo-, serine, acid and sulfhydryl proteases and their inhibitors. Protease activity is indicated by increasing green fluorescence (excitation/emission maxima ~503/513 nm).
EnzChek Protease Assay Kit *red fluorescence*	(R-22132)	(R-22133)	A direct fluorescence-based assay for detecting metallo-, serine, acid and sulfhydryl proteases and their inhibitors. Protease activity is indicated by increasing red fluorescence (excitation/emission maxima ~589/617 nm).
EnzChek Tyrosine Phosphatase Assay Kit	(R-22067)	(R-22068)	An assay for monitoring protein tyrosine phosphatases (PTP) and for screening PTP inhibitors and modulators. PTP activity is indicated by increasing blue fluorescence (excitation/emission maxima ~358/452 nm).
EnzChek Serine/Threonine Phosphatase Assay Kit	(R-33700)	Inquire	An assay for monitoring serine/threonine (Ser/Thr) phosphatases and for screening Ser/Thr inhibitors and modulators. Ser/Thr phosphatase activity is indicated by increasing blue fluorescence (excitation/emission maxima ~358/452 nm).
PicoGreen dsDNA Assay Kit	(R-21495)	Inquire	An assay for accurately quantitating low levels of dsDNA (250 pg/mL) with a broad dynamic range — over four orders of magnitude. When bound to nucleic acids, the PicoGreen reagent exhibits excitation/emission maxima of ~502/523 nm.

RiboGreen RNA Quantitation Kit	(R-32700)	Inquire	An assay for accurately quantitating low levels of RNA (1 ng/mL) with a broad dynamic range — over three orders of magnitude using two different dye concentrations. When bound to RNA, the RiboGreen reagent exhibits excitation/emission maxima of ~500/520 nm.

* * * * *

Table 10.4 — Detection limits of the EnzChek Protease Assay Kits (E-6638, E-6639)

Enzyme (Source)	Class	Detection Limit (Units) *	Buffer Conditions
Elastase, Type IV (porcine pancreas)	Serine protease	2.2×10^{-3}	10 mM Tris-HCl, pH 8.8
Chymotrypsin, Type II (bovine pancreas)	Serine protease	5.0×10^{-5}	10 mM Tris-HCl, pH 7.8
Thermolysin (*Bacillus thermoproteolyticus* Rokko)	Acid protease	4.4×10^{-5}	10 mM Tris-HCl, pH 7.8
Trypsin, Type IX (porcine pancreas)	Serine protease	1.3×10^{-2}	10 mM Tris-HCl, pH 7.8
Papain (papaya latex)	Cysteine protease	2.1×10^{-4}	10 mM MES, pH 6.2
Pepsin (porcine stomach mucosa)	Acid protease	2.1×10^{-3}	10 mM HCl, pH 2.0
Elastase (*Pseudomonas aeruginosa*)	Metallo-protease	1.0×10^{-3}	20 mM sodium phosphate, pH 8.0
Cathepsin D	Acid protease	2.0×10^{-4}	20 mM sodium citrate, pH 5.0
Elastase (human leukocyte)	Serine protease	1.0×10^{-3}	10 mM Tris-HCl, pH 7.5

* The detection limit is defined as the amount of enzyme required to cause a 10–20% change in fluorescence compared to the control sample at 22°C. Enzyme unit definitions are standard definitions for each individual enzyme. Detection limits were determined with BODIPY FL casein and with BODIPY TR-X casein; both substrates yielded similar results. Detection limits may vary with instrumentation.

* * * * *

Product List — 10.4 Detecting Peptidases and Proteases

Cat #	Pr duct Name	Unit Size
A-6520	7-amino-4-chloromethylcoumarin, *t*-BOC-L-leucyl-L-methionine amide (CMAC, *t*-BOC-Leu-Met)	5 mg
A-22127	7-amino-4-methylcoumarin, *N*-CBZ-L-isoleucyl-L-glutamyl-L-	5 mg

	threonyl-L-aspartic acid amide (Z-IETD-AMC)	
A-6521	7-amino-4-methylcoumarin, CBZ-L-phenylalanyl-L-arginine amide, hydrochloride	25 mg
A-22121	7-amino-4-trifluoromethylcoumarin, *N*-CBZ-L-aspartyl-L-glutamyl-L-valyl-L-aspartic acid amide (Z-DEVD-AFC)	5 mg
A-22128	7-amino-4-trifluoromethylcoumarin, *N*-CBZ-L-isoleucyl-L-glutamyl-L-threonyl-L-aspartic acid amide (Z-IETD-AFC)	5 mg
A-21290	anti-matrix metalloproteinase-1 (hinge region), affinity-purified rabbit antibody (anti-MMP-1) *1 mg/mL*	100 µL
A-21291	anti-matrix metalloproteinase-2 (hinge region), affinity-purified rabbit antibody (anti-MMP-2) *1 mg/mL*	100 µL
A-21292	anti-matrix metalloproteinase-3 (hinge region), affinity-purified rabbit antibody (anti-MMP-3) *1 mg/mL*	100 µL
A-21293	anti-matrix metalloproteinase-9 (hinge region), affinity-purified rabbit antibody (anti-MMP-9) *1 mg/mL*	100 µL
C-2990	casein, fluorescein conjugate	25 mg
D-12060	DQ™ collagen, type I from bovine skin, fluorescein conjugate	1 mg
D-12052	DQ™ collagen, type IV from human placenta, fluorescein conjugate	1 mg
D-12054	DQ™ gelatin from pig skin, fluorescein conjugate *special packaging*	5 x 1 mg
D-12050	DQ™ Green BSA *special packaging*	5 x 1 mg
D-12053	DQ™ ovalbumin *special packaging*	5 x 1 mg
D-12051	DQ™ Red BSA *special packaging*	5 x 1 mg
E-13183	EnzChek® Caspase-3 Assay Kit #1 *Z-DEVD-AMC substrate* *500 assays*	1 kit
E-13184	EnzChek® Caspase-3 Assay Kit #2 *Z-DEVD-R110 substrate* *500 assays*	1 kit
E-12056	EnzChek® Elastase Assay Kit *600 assays*	1 kit
E-12055	EnzChek® Gelatinase/Collagenase Assay Kit *250-2000 assays*	1 kit
E-6658	EnzChek® Polarization Assay Kit for Proteases *green fluorescence* *100-1000 assays*	1 kit
E-6639	EnzChek® Protease Assay Kit *green fluorescence* *100-1000 assays*	1 kit
E-6639	EnzChek® Protease Assay Kit *red fluorescence* *100-1000 assays*	1 kit
H-2990	HIV Protease Substrate 1 (Arg-Glu(EDANS)-Ser-Gln-Asn-Tyr-Pro-Ile-Val-Gln-Lys(dabcyl)-Arg)	1 mg
P-12271	pepstatin A, BODIPY® FL conjugate	25 µg
R-35100	RediPlate™ 96 EnzChek® Caspase-3 Assay Kit *Z-DEVD-R110 substrate* *one 96-well microplate*	1 kit
R-22120	RediPlate™ 96 EnzChek® Protease Assay Kit *green fluorescence* *one 96-well microplate*	1 kit
R-22121	RediPlate™ 384 EnzChek® Protease Assay Kit *green fluorescence* *one 384-well microplate*	1 kit
R-22132	RediPlate™ 96 EnzChek® Protease Assay Kit *red fluorescence* *one 96-well microplate*	1 kit

R-2213[illegible]	RediPlate™ 384 EnzChek® Protease Assay Kit *red fluorescence* *one 384-well microplate*	1 kit
R-2931	Renin Substrate 1 (Arg-Glu(EDANS)-Ile-His-Pro-Phe-His-Leu-Val-Ile-His-Thr-Lys(dabcyl)-Arg)	1 mg
R-2212[illegible]	rhodamine 110, bis-(L-aspartic acid amide), trifluoroacetic acid salt	1 mg
R-6504	rhodamine 110, bis-(CBZ-L-alanyl-L-alanine amide)	5 mg
R-6505	rhodamine 110, bis-(CBZ-L-alanyl-L-alanyl-L-alanyl-L-alanine amide)	5 mg
R-6503	rhodamine 110, bis-(CBZ-L-alanyl-L-arginine amide), dihydrochloride	5 mg
R-6501	rhodamine 110, bis-(CBZ-L-arginine amide), dihydrochloride (BZAR)	5 mg
R-22120	rhodamine 110, bis-(*N*-CBZ-L-aspartyl-L-glutamyl-L-valyl-L-aspartic acid amide) (Z-DEVD-R110) *bulk packaging*	20 mg
R-2212[illegible]	rhodamine 110, bis-(*N*-CBZ-L-isoleucyl-L-glutamyl-L-threonyl-L-aspartic acid amide) (Z-IETD-R110)	2 mg
R-2212[illegible]	rhodamine 110, bis-(*N*-CBZ-L-isoleucyl-L-glutamyl-L-threonyl-L-aspartic acid amide) (Z-IETD-R110) *bulk packaging*	20 mg
R-6505	rhodamine 110, bis-(CBZ-L-isoleucyl-L-prolyl-L-arginine amide), dihydrochloride (BZiPAR)	5 mg
R-6502	rhodamine 110, bis-(CBZ-L-phenylalanyl-L-arginine amide), dihydrochloride	5 mg
R-3371[illegible]	rhodamine 110, bis-(*N*-CBZ-L-alanyl-L-alanyl-L-aspartic acid amide) (Z-AAD-R110)	2 mg
R-337[illegible]	rhodamine 110, bis-(*N*-CBZ-L-leucyl-L-glutamyl-L-glutamyl-L-aspartic acid amide) (Z-LEED-R110)	2 mg
R-33751	rhodamine 110, bis-(*N*-CBZ-L-leucyl-L-glutamyl-L-histidinyl-L-aspartic acid amide) (Z-LEHD-R110)	2 mg
R-33750	rhodamine 110, bis-(*N*-CBZ-L-tyrosinyl-L-valyl-L-alanyl-L-aspartic acid amide) (Z-YVAD-R110)	2 mg
R-33755	rhodamine 110, bis-(*N*-CBZ-L-valyl-L-aspartyl-L-valyl-L-alanyl-L-aspartic acid amide) (Z-VDVAD-R110)	2 mg
R-3374[illegible]	rhodamine 110, bis-(*N*-CBZ-L-valyl-L-glutamyl-L-isoleucyl-L-aspartic acid amide) (Z-VEID-R110)	2 mg
R-22123	rhodamine 110, bis-(succinoyl-L-alanyl-L-alanyl-L-prolyl-L-phenylalanyl amide)	1 mg
R-2212[illegible]	rhodamine 110, bis-(p-tosyl-L-glycyl-L-prolyl-L-arginine amide)	2 mg
T-2[illegible]	trypsin inhibitor from soybean, Alexa Fluor® 488 conjugate	1 mg

* * * * *

Data Table — 10.4 Detecting Peptidases and Proteases

Cat #	MW	Storage	Soluble	Abs	EC	Em	Solvent	Product	Notes
A-6[illegible]	554.10	F,D	DMSO	330	13,000	403	MeOH	C-2110 (&item=2110">see data)	1

A-65 1	649.14	F,D	DMSO	326	19,000	384	MeOH	A-191 (see data)	1
A-65 75	536.41	F,D	DMSO	330	15,000	403	MeOH	C-2110 (see data)	1
A-22 21	821.72	F,D	DMSO	339	7,900	435	MeOH	see Notes	1, 2
A-22 27	767.79	F,D	DMSO	326	17,000	392	MeOH	A-191 (see data)	1
A-22 8	821.76	F,D	DMSO	340	8,200	434	MeOH	see Notes	1, 2
D-62 1	477.31	F,D,L	DMF, MeCN	503	84,000	510	MeOH		
E-13 83	767.74	F,D,L	DMSO	326	16,000	387	MeOH	A-191 (&item=191">see data)	1, 3
E-13 4	1515.46	F,D,L	DMSO	232	52,000	none	MeOH	R-6479 (see data)	3
H-29	~2016	F,D,L	DMF, H_2O	430	23,000	none	MeOH	see Notes	4
L-65 3	~475	F,D	H_2O	<300		none			
P-65 42	685.90	F,D	DMSO, MeOH	<300		none			
P-12 71	1044.14	F,D,L	DMSO	504	86,000	511	MeOH		
R-2	~2281	F,D,L	DMF, H_2O	460	13,000	none	H_2O	see Notes	4
R-65 1	983.91	F,D	DMSO, DMF	232	55,000	none	MeOH	R-6479 (&item=6479">see data)	
R-65 02	1278.26	F,D	DMSO, DMF	232	60,000	none	MeOH	R-6479 (&item=6479">see data)	
R-6 3	882.92	F,D	DMSO, DMF	232	57,000	none	MeOH	R-6479 (&item=6479">see data)	
R-65 05	1404.46	F,D	DMSO, DMF	231	44,000	none	MeOH	R-6479 (&item=6479">see data)	
R-6 6	1167.24	F,D	DMSO, DMF	232	56,000	none	MeOH	R-6479 (&item=6479">see data)	
R-6 7	1178.14	F,D	DMSO, DMF	232	58,000	none	MeOH	R-6479 (&item=6479">see data)	
R-6 8	1126.06	F,D	DMSO, DMF	232	57,000	none	MeOH	R-6479 (&item=6479">see data)	
R-6 3	1047.33	F,D	DMSO, DMF	232	60,000	none	MeOH	R-6479 (&item=6479">see data)	
R-6 55	687.58	F,D	DMSO,	232	57,000	none	MeOH	see Notes	5

			DMF						
R-6 7	632.54	F,D	DMSO, DMF	232	58,000	none	MeOH	see Notes	5
R-6' 0	809.70	F,D	DMSO, DMF	232	54,000	none	MeOH	see Notes	5
R-6. '	852.74	F,D	DMSO, DMF	232	56,000	none	MeOH	R-6479 (&item=6479">see data)	
R-2 . 20	1515.46	F,D	DMSO, DMF	232	52,000	none	MeOH	R-6479 (&item=6479">see data)	
R-2 22	788.57	F,D	DMSO, DMF	232	55,000	none	MeOH	R-6479 (&item=6479">see data)	
R-22 23	1303.39	F,D	DMSO, DMF	232	53,000	none	MeOH	R-6479 (&item=6479">see data)	
R-2 24	1259.42	F,D	DMSO, DMF	232	54,000	none	MeOH	R-6479 (&item=6479">see data)	
R-22 25	1515.55	F,D	DMSO, DMF	232	52,000	none	MeOH	R-6479 (&item=6479">see data)	
R-2 26	1515.55	F,D	DMSO, DMF	232	52,000	none	MeOH	R-6479 (&item=6479">see data)	
R-3 30	1495.56	F,D	DMSO, DMF	230	76,000	none	MeOH	R-6479 (&item=6479">see data)	
R-3: 51	1587.62	F,D	DMSO, DMF	232	57,000	none	MeOH	R-6479 (&item=6479">see data)	
R-3: 52	1113.10	F,D	DMSO, DMF	232	57,000	none	MeOH	R-6479 (&item=6479">see data)	
R-3 3	1571.57	F,D	DMSO, DMF	232	57,000	none	MeOH	R-6479 (&item=6479">see data)	
R-3 54	1511.60	F,D	DMSO, DMF	232	57,000	none	MeOH	R-6479 (&item=6479">see data)	
R-3 55	1597.65	F,D	DMSO, DMF	232	57,000	none	MeOH	R-6479 (&item=6479">see data)	

Not

1. .orescence of the unhydrolyzed substrate is very weak.

2. Enzymatic cleavage of this substrate yields 7-amino-4-trifluoromethylcoumarin: Abs = 380 nm (ε = 13,000 $cm^{-1}M^{-1}$), Em = 500 nm in pH 7 buffer.
3. Data represent the substrate component of this kit.
4. Fluorescence of this substrate is >99% quenched. The proteolytic cleavage products fluoresce at 500 nm (excitation at 335 nm).
5. Peptidase cleavage product is a 4-(chloromethyl)benzoyl amide derivative of rhodamine 110, Abs = 492 nm (EC = 24,000 $cm^{-1}M^{-1}$), Em = 529 nm in MeOH (based on reported data) (Biochem J 209, 299 (1983)).

* * * * *

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Section 10.5 — Substrates for Oxidases, Including Amplex Red Kits

Oxidases, the most useful of which is undoubtedly horseradish peroxidase (HRP), are important enzymes that are used in a wide variety of bioassays. Peroxidase activity is also present in many cells. Reagents for quantitation of peroxidase and the activity of a variety of other oxidases are described in this section; reagents for detecting the activity of cellular peroxidases and the oxygen radicals produced by these peroxidases are described in Section 16.1 and Section 19.2. Antibody, protein G, avidin and streptavidin conjugates of horseradish peroxidase are listed in the price list of this section and described in Section 7.3 and Section 7.6. Tyramide signal-amplification (TSA) technology (Section 6.2) makes extensive use of peroxidase-conjugated reagents and fluorescent dye– or hapten-labeled tyramides to deposit a detectable product at the site of enzymatic activity (Figure 6.5). Our exclusive Zenon technology (Section 7.2) includes the Zenon Horseradish Peroxidase Antibody Labeling Kits (Table 7.1), which permit the rapid and quantitative formation of HRP-labeled antibody complexes. The Zenon Antibody Labeling Kits and the Zenon technology are described in detail in Section 7.2.

We have used our extremely versatile Amplex Red reagent — the most stable and sensitive fluorogenic substrate known for horseradish peroxidase — to develop a variety of novel fluorogenic and chromogenic assays for enzymes that produce hydrogen peroxide. Furthermore, these coupled assays permit the ultrasensitive quantitation of a diverse assortment of analytes, including glucose, galactose, cholesterol, glutamic acid, xanthine (or hypoxanthine), uric acid, choline and acetylcholine, as well as hydrogen peroxide. Our patented P_iPer Phosphate Assay Kit and P_iPer Pyrophosphate Assay Kit (P-22061, P-22062; Section 10.3) also utilize our exclusive Amplex Red technology for the continuous assays of enzymes that produce either inorganic phosphate or pyrophosphate.

Peroxidases

Amplex Red Reagent: A Stable Substrate for Peroxidase Detection

The best fluorogenic substrate known for peroxidase and any other oxidase that produces H_2O_2 is the Amplex Red reagent (10-acetyl-3,7-dihydroxyphenoxazine; A-12222, A-22177; Figure 10.49), which is a sensitive and stable probe for H_2O_2. In the presence of horseradish peroxidase (HRP), the Amplex Red reagent reacts with a 1:1 stoichiometry with H_2O_2 to produce highly fluorescent resorufin (R-363, Figure 10.50, Section 10.1). Unlike most peroxidase substrates, the Amplex Red reagent is very stable in the presence of H_2O_2, except when a peroxidase is present. The sensitivity of the Amplex Red reagent in detecting the activity of D-amino acid oxidase has been reported to be 5- to 5-times better than that of the QuantaBlu fluorogenic peroxidase substrate,[2] with a lower limit of detection of D-alanine of 2 pmol.[2] The Amplex Red reaction can be used to routinely detect as little as 10 picomoles of H_2O_2 in a 100 µL volume (50 nM, Figure 10.51), at least a tenfold greater sensitivity than that attained with the commonly used scopoletin assay for H_2O_2.[1,3] In the scopoletin assay, HRP catalyzes conversion of the fluorescent scopoletin to a nonfluorescent product. Unlike scopoletin, the Amplex Red reagent is a fluorogenic substrate with very low background fluorescence. Consequently, assays using Amplex Red as the substrate result in an increase in fluorescence, not as a decrease — an inherently superior method for enzymatic assays. Other advantages of the Amplex Red reaction over scopoletin-based H_2O_2 assays include high chemical stability of the Amplex Red reagent and its fluorescent product, resorufin, and the long wavelength spectra of resorufin. Because resorufin has excitation and fluorescence emission maxima (Figure 10.5) of approximately 570 nm and 585 nm (versus 360 nm and 460 nm,

resp ·ly, for scopoletin), there is much less interference from autofluorescence in most biolo· samples.

Bec· H_2O_2 is produced in many different enzymatic reactions, the Amplex Red reagent can be used ·oupled enzymatic reactions to detect the activity of many different enzymes or, when the subs·· e concentration is limited, to assay solutions for metabolically active constituents such as gluco· acetylcholine and cholesterol (see below). Advantages of Amplex Red reagent–based assay ·clude the following:

- T Amplex Red reagent is a fluorogenic substrate with extremely low background color or f ·scence
- S k solutions of the Amplex Red reagent are chemically stable
- f ·orescent product, resorufin, is also stable
- long-wavelength spectra of resorufin (excitation/emission ~570/585 nm, Figure 10.5) r ·t in little interference from autofluorescence in most biological samples

The A· ·ex Red reagent has been used to detect the release of H_2O_2 from activated human leukoc s,[1,3] to measure the activity of monoamine oxidase in cow brain tissue,[4] to demonstrate the ex ·cellular production of H_2O_2 produced by UV light stimulation of human keratinocytes [5–7] and t· ·asure L-glutamate in food samples.[8] The Amplex Red reagent can be purchased sepa· in a single 5 mg vial (A-12222) or packaged as a set of 10 vials, each containing 10 mg of th· ·strate, for high-throughput screening applications (A-22177). The Amplex Red reagent is also u ·ed as the detection reagent in our many Amplex Red Assay Kits, including the:

- A ·ex Red Hydrogen Peroxide/Peroxidase Assay Kit (A-22188)
- A· ·ex Red Catalase Assay Kit (A-22180)
- A ·x Red Monoamine Oxidase Assay Kit (A-12214)
- A· ·x Red Glutamic Acid/Glutamate Oxidase Assay Kit [8] (A-12221)
- A ·x Red Glucose/Glucose Oxidase Assay Kit (A-22189)
- A ·x Red Galactose/Galactose Oxidase Assay Kit (A-22179)
- A ·ex Red Neuraminidase (Sialidase) Assay Kit (A-22178)
- A ·ex Red Cholesterol Assay Kit (A-12216)
- A ·ex Red Acetylcholine/Acetylcholinesterase Assay Kit (A-12217)
- A ·x Red Phosphatidylcholine-Specific Phospholipase C Assay Kit (A-12218, Section 18.4)
- A· ·ex Red Phospholipase D Assay Kit (A-12219, Section 18.4)
- A .x Red Sphingomyelinase Assay Kit (A-12220, Section 13.3)
- A ·x Red Uric Acid/Uricase Assay Kit (A-22181)
- A x Red Xanthine/Xanthine Oxidase Assay Kit (A-22182)
- A .x Red ELISA Kits #1 and #2 (A-22170, A-22171)

Most ·· Amplex Red kits are further discussed in this section; however, some are only prese· in the sections listed above. The Amplex Red reagent and kits containing the Amplex Red reage e sold for noncommercial use and for high-throughput screening applications only.

Amp· ·x Red ELISA Kits

Molec · robes' Amplex Red ELISA Kits offer an extremely sensitive fluorometric or colorimetric detec ethod for horseradish peroxidase (HRP)–amplified enzyme-linked immunosorbent assay ·SAs). The Amplex Red ELISA Kit #1 (A-22170) contains an HRP goat anti-mouse IgG conju · which can be used for the ELISA detection of any mouse IgG antibody. The Amplex Red ELISA ·2 (A-22171) contains the versatile protein G conjugate of HRP, which can be used for the E detection of IgGs from most commonly used species, including human, mouse, rabbit, goat, ·eep, cow and horse. The Amplex Red reagent provided in these kits is a highly sensitive and st ble probe for the detection of HRP activity. As described above, the Amplex Red reagent reacts ·it· H_2O_2 in the presence of HRP to form the fluorescent product resorufin [1,3] (R-363,

Sectio 0.1, Figure 10.50). Because resorufin also has strong absorption, the assay can be perfo d either fluorometrically or spectrophotometrically. The Amplex Red ELISA Kit #1 with the HRP g nti-mouse IgG antibody conjugate has detection limits of as little as 10 pg/microplate well c ouse monoclonal IgG by fluorometry or 50 pg/microplate well by colorimetry (Figure 7.56). Amplex Red ELISA Kit #2 with HRP protein G has detection limits of as little as 1 ng/mi ate well of a mouse monoclonal IgG by fluorometry or 3 ng/microplate well by colorir t .

The Am lex Red ELISA Kits contain:

- T Amplex Red reagent
- D hylsulfoxide (DMSO)
- entrated reaction buffer
- en peroxide (H_2O_2)
- A eradish peroxidase (HRP) conjugate of goat anti-mouse IgG antibody (in Kit #1, A 170) or protein G (in Kit #2, A-22171)
- De ed ELISA protocols

The Ar lex Red ELISA Kits provide sufficient reagents for approximately 1000 ELISAs using either a fluc nce- or absorbance-based microplate reader and reaction volumes of 100 µL per assay. Our H njugates of goat anti-mouse IgG antibody (G-21040, Section 7.3) and protein G (P-2 Section 7.3) are also available separately.

Othe strates for Peroxidase Assays

Althou RP is an important enzyme for both histochemistry and ELISAs, fluorogenic peroxidase substr have not been extensively used for its detection. Fluorogenic peroxidase substrates such as the ydrofluoresceins (also known as fluorescins) (D-399, C-400, C-13293), dihydrocalcein AM (D-23 Figure 15.4), dihydrorhodamines (D-632, D-633, D-23806; Section 19.3) and dihyd dium (hydroethidine; D-1168, D-11347, D-23107; Section 19.2) are converted to highly fluor products in the presence of the enzyme and hydrogen peroxide. Because these subs are insufficiently stable for routine use in ELISA assays, Molecular Probes has converted the di fluoresceins to diacetates. When used in intracellular applications, the acetates are cleav endogenous esterases, releasing the intact substrate. However, when used for *in vitro* assays esterase or a mild base must first be added to cleave the acetates, releasing the substr The dihydrofluoresceins have been used to measure peroxidase activity [9] and to detect hydr xide formation.[10–13] In addition to being a reagent for derivatization of aldehydes and ketone ection 3.2) and detection of nitric oxide (Section 19.3), NBD methylhydrazine (*N*-me 4-hydrazino-7-nitrobenzofurazan, M-20490) has been reported to be useful as a fluoro peroxidase substrate, with a sensitivity limit for detection of H_2O_2 of about 75 nM.[14]

Lur d MCLA: Chemiluminescent Peroxidase Substrates

Nonis c immunoassays utilizing peroxidase conjugates and the chemiluminescent horseradish perc substrate luminol (L-8455) have provided a rapid and sensitive method for quantitating a wi iety of analytes, including cholesterol,[15] digoxin [16] and acetylcholine.[17] Addition of trace am ur luciferin (L-2911, L-2912, L-2916; Section 10.6) has been shown to considerably enhan e sensitivity in the assay of thyroxine, digoxin, α-fetoprotein and other analytes.[18] A metho t employs luminol has been developed for the quantitation of very limiting samples of human A from single hairs, saliva, small blood stains and paraffin-embedded and fixed tissue sect ing a biotinylated oligodeoxynucleotide probe to membrane-immobilized DNA, a strep n–horseradish peroxidase conjugate and luminol, researchers have detected 150 pg of hum A.[19]

MC 3800) is principally utilized as a superoxide-sensitive chemiluminescent probe (Section

19.[illegible] ...A has also been utilized for the determination of both horseradish peroxidase [20] and my...lo... ...xidase.[21-23]

Am...l... Red Hydrogen Peroxide/Peroxidase Assay Kit

The ...x Red Hydrogen Peroxide/Peroxidase Assay Kit (A-22188) provides a simple, one-step ass... ...letecting H_2O_2 or the activity of horseradish peroxidase using a fluorescence-based mic...p... reader or a fluorometer (Figure 10.53). Using the reagents in the kit one can also assay the ...c... ...y of other oxidases that produce H_2O_2, such as NADPH oxidase.[24] This assay has been use... t... ...tect H_2O_2 release from activated human leukocytes.[3] In the scopoletin assay, HRP cat...v... the conversion of fluorescent scopoletin to a nonfluorescent product. Unlike scopoletin, the ...n... ...x Red reagent is a fluorogenic substrate with very low background fluorescence. Other advanta... ...s of the Amplex Red reaction over scopoletin-based H_2O_2 assays include high chemical stability ...f the Amplex Red reagent and its fluorescent product, resorufin, and the long wavelength spectra o... resorufin. Because resorufin has excitation and fluorescence emission maxima (Figure 10.5) of ...proximately 570 nm and 585 nm (versus 360 nm and 460 nm, respectively, for scopolet... , there is much less interference from autofluorescence in most biological samples. The Amplex R...d Hydrogen Peroxide Assay Kit contains the principal detection reagents required to assay m...ny other oxidases that produce H_2O_2, such as NADPH oxidase.[24] Included in the Amplex Red Hyd...gen Peroxide/Peroxidase Assay Kit are:

- The ...mplex Red reagent
- Hors...radish peroxidase (HRP)
- H_2O... for use as a positive control
- DMS... and a concentrated reaction buffer
- A d...ailed protocol

Each kit ...rovides a sufficient amount of each reagent for approximately 500 assays using a reaction vol...me ... 100 µL per assay.

Catalas...

The Amp...x Red Catalase Assay Kit (A-22180) provides an ultrasensitive, yet simple, assay for measuri... catalase activity. Catalase is a heme-containing redox protein found in nearly all animal and plan...cells as well as in aerobic microorganisms. In eukaryotic cells it is concentrated in the peroxiso...es. Catalase is an important enzyme because H_2O_2 is a powerful oxidizing agent that is potential...damaging to cells. By preventing excessive buildup of H_2O_2, catalase allows important cel...ular ...ocesses that produce H_2O_2 as a by-product to take place safely.

In the as...y, catalase first reacts with H_2O_2 to produce water and oxygen (O_2). Next, the Amplex Red reag...t reacts with a 1:1 stoichiometry with any unreacted H_2O_2 in the presence of ho...serac... peroxidase to produce the highly fluorescent oxidation product, resorufin. Therefore, as catalase ...tivity increases, the signal from resorufin decreases (Figure 10.54). The results are typically ...lotted by subtracting the observed fluorescence from that of a no-catalase control. Using th... kit, ...s possible to detect catalase in a purified system at levels as low as 50 mU/mL.

Re...orufi... has absorption and fluorescence emission maxima of approximately 563 nm and 587 nm, respectiv...ly (Figure 10.5). Because the absorbance is strong, the assay can be performed either fluorome...cally or spectrophotometrically. The Amplex Red Catalase Assay Kit contains

- The ...mplex Red reagent
 Hor...adish peroxidase (HRP)

- Catalase
- H_2O_2
- DMSO and a concentrated reaction buffer
- A detailed protocol

Each kit provides sufficient reagents for approximately 400 assays using either a fluorescence- or absorbance-based microplate reader and reaction volumes of 100 µL per assay.

Glucose and Glucose Oxidase

Glucose oxidase is widely used for glucose determination and, when conjugated to antibodies, for use in enzyme immunoassays (EIAs). Molecular Probes has found that the Amplex Red reagent can be utilized for the ultrasensitive detection of both glucose and glucose oxidase. In this enzyme-coupled assay, glucose oxidase reacts with glucose to form gluconolactone and H_2O_2. The H_2O_2 is then detected using the Amplex Red reagent peroxidase substrate (Figure 10.50). The Amplex Red Glucose/Glucose Oxidase Assay Kit (A-22189) can be used to detect glucose levels as low as 3 µM or 0.5 µg/mL (Figure 10.23) and is at least tenfold more sensitive than assays using *o*-dianisidine as the peroxidase substrate. The same kit can be used to detect glucose oxidase levels as low as 0.05 mU/mL (Figure 10.55). This Amplex Red Kit can be used with a fluorescence-based microplate reader or a fluorometer and contains:

- The Amplex Red reagent
- Horseradish peroxidase (HRP)
- H_2O_2 for use as a positive control
- DMSO and a concentrated reaction buffer
- D-Glucose
- Glucose oxidase
- A detailed protocol

The kit provides a sufficient amount of each reagent for approximately 500 assays using a reaction volume of 100 µL per assay.

Galactose and Galactose Oxidase

The Amplex Red Galactose/Galactose Oxidase Assay Kit (A-22179) provides the reagents and a general protocol for the assay of terminal galactosylated proteins, galactose-producing enzymes and for the assay of galactose oxidase. Unlike glucose oxidase, galactose oxidase can produce H_2O_2 from either free galactose or from polysaccharides — including glycoproteins in solution or on cell surfaces — in which galactose is the terminal residue, producing an aldehyde moiety on the 6-position of the galactose (Figure 10.13). We have used our Amplex Red galactose oxidase assay for the quantitative assay of mucin-type glycoproteins by using a method similar to one described by Kinosita and collaborators.[25] The Amplex Red Galactose/Galactose Oxidase Assay Kit (A-22179) contains:

- The Amplex Red reagent
- Dimethylsulfoxide (DMSO)
- Galactose
- Galactose oxidase from *Dactylium dendroides*
- Horseradish peroxidase (HRP)
- H_2O_2
- A 5x reaction buffer
- A detailed protocol for the assay

Sufficient reagents are provided for approximately 400 assays using either a fluorescence- or

absorption-based microplate reader and reaction volumes of 100 µL per assay. The Amplex Red fluorescent galactose/galactose oxidase assay can accurately detect free galactose concentration as low as 4 µM (Figure 10.14) and 2 mU/mL of galactose oxidase activity (Figure 10.15). Because of the high absorbance of resorufin, the absorptimetric assay has only slightly lower sensitivity. The Amplex Red Neuraminidase (Sialidase) Assay Kit (A-22178) utilizes this galactose oxidase–coupled chemistry for continuous assay of neuraminidase-catalyzed hydrolysis of fetuin, a sialoglyconjugate. This product is described in detail in Section 10.2.

Ch lesterol and Cholesterol Oxidase

The Amplex Red Cholesterol Assay Kit (A-12216) provides what may be the most sensitive assay available for cholesterol in serum and other complex mixtures. The assay provided in this kit can detect as little as 5 ng/mL (5×10^{-4} mg/dL) cholesterol (Figure 13.39) and can accurately measure the cholesterol content in the equivalent of 0.01 µL of human serum.[26] In the assay, cholesterol is detected by an enzyme-coupled reaction. Cholesteryl esters are hydrolyzed by cholesterol esterase into cholesterol, which is then oxidized by cholesterol oxidase to yield H_2O_2 and the corresponding ketone product. The H_2O_2 is then detected using the Amplex Red reagent. The Amplex Red cholesterol assay is continuous, requires no separation or wash steps and, except potentially at extremely high concentrations, is insensitive to bilirubin contamination. These characteristics make the assay particularly well suited for the rapid and direct analysis of cholesterol in blood and food samples using automated instruments. By performing reactions in the presence and absence of cholesterol esterase, the assay is also potentially useful for determining the fraction of cholesterol that is in the form of cholesteryl esters within a sample. In addition, by providing an excess of cholesterol in the reaction, the assay can be used to sensitively detect the activity of cholesterol oxidase. This kit can be used with a fluorescence-based microplate reader or a fluorometer and contains:

- The Amplex Red reagent
- Horseradish peroxidase (HRP)
- H_2O_2 for use as a positive control
- DMSO and a concentrated reaction buffer
- Cholesterol oxidase
- Cholesterol esterase
- Cholesterol for preparation of a standard curve
- A detailed protocol

Each kit provides a sufficient amount of reagent for approximately 500 assays using a reaction volume of 100 µL per assay.

Monoamine Oxidase

Monoamine oxidase, which inactivates several primary, secondary and tertiary amines via oxidative transamination, serves to regulate tissue levels of amine neurotransmitters and dietary amines. The Amplex Red Monoamine Oxidase Assay Kit (A-12214) provides a simple fluorometric method for the continuous measurement of amine oxidase activity in tissue homogenates or purified preparations. We have found that the assay is able to sensitively detect both monoamine oxidase (MAO) activity and semicarbazide-sensitive amine oxidase (SSAO) activity and is useful for performing both end-point and continuous measurements of amine oxidase activity. The assay is able to detect both MAO-A and MAO-B from cow brain tissue using as little as 200 µg of total protein per sample [4] and has been used to measure plasma amine oxidase (an SSAO) activity levels as low as 1.2×10^{-5} U/mL using a commercially available enzyme (Figure 10.56).

To facilitate discrimination of MAO-A and MAO-B activity, two MAO substrates and two MAO inhibitors are included in the kit. *p*-Tyramine is a substrate for both MAO-A and MAO-B, whereas benzylamine is a substrate for MAO-B.[27] Both *p*-tyramine and benzylamine are also substrates for

SSAO enzymes. Clorgyline is a specific inhibitor of MAO-A activity and pargyline is a specific inhibitor of MAO-B activity.[28,29] The potential applications of this kit include the measurement of amine oxidase activity in normal and diseased tissues, blood samples and other biological fluids, the screening of drugs as possible MAO inhibitors or substrates and the determination of kinetic constants for different amine oxidase substrates. Each kit contains:

- The Amplex Red reagent
- Horseradish peroxidase (HRP)
- H_2O_2 for use as a positive control
- DMSO and a concentrated reaction buffer
- Benzylamine, a substrate for MAO-B and SSAO enzymes
- *p*-Tyramine, a substrate for MAO-A, MAO-B and SSAO enzymes
- Clorgyline, a specific inhibitor of MAO-A activity
- Pargyline, a specific inhibitor of MAO-B activity
- Resorufin for use as a reference standard (Figure 10.5)
- A detailed protocol

Each kit provides a sufficient amount of each reagent for approximately 500 assays using a reaction volume of 200 µL per assay.

Glutamic Acid and Glutamate Oxidase

The Amplex Red Glutamic Acid/Glutamate Oxidase Assay Kit (A-12221) provides an ultrasensitive method for continuously detecting glutamic acid [30] or for monitoring glutamate oxidase activity in a fluorescence-based microplate reader or a fluorometer.[8] In this assay, L-glutamic acid is oxidized by glutamate oxidase to produce α-ketoglutarate, NH_3 and H_2O_2. L-Alanine and L-glutamate–pyruvate transaminase are also included in the reaction. Thus, the L-glutamic acid is regenerated by transamination of α-ketoglutarate, resulting in multiple cycles of the initial reaction and a significant amplification of the H_2O_2 produced. Hydrogen peroxide reacts with the Amplex Red reagent in a 1:1 stoichiometry in a reaction catalyzed by horseradish peroxidase (HRP) to generate the highly fluorescent product resorufin [1,3] (R-363, Section 10.1). Because resorufin has absorption/emission maxima of 563/587 nm (Figure 10.5), there is little interference from autofluorescence in most biological samples

If the concentration of L-glutamic acid is limiting in this assay, then the fluorescence increase is proportional to the initial L-glutamic acid concentration. The Amplex Red Glutamic Acid/Glutamate Oxidase Assay Kit allows detection of as little as 10 nM L-glutamic acid in purified systems using a 30-minute reaction time (Figure 16.36). If the reaction is modified to include an excess of L-glutamic acid, then this kit can be used to continuously monitor glutamate oxidase activity. For example, purified L-glutamate oxidase from *Streptomyces* can be detected at levels as low as 40 µU/mL (Figure 16.37).

The Amplex Red reagent has been used to quantitate the activity of glutamate-producing enzymes in a high-throughput assay for drug discovery.[30] Each Amplex Red Glutamic Acid/Glutamate Oxidase Assay Kit contains:

- The Amplex Red reagent
- Horseradish peroxidase (HRP)
- H_2O_2
- DMSO and a concentrated reaction buffer
- L-Glutamate oxidase from *Streptomyces* sp.
- L-Glutamate–pyruvate transaminase from pig heart
- L-Glutamic acid
- L-Alanine

- A detailed protocol

Each kit provides sufficient reagents for approximately 200 assays using a fluorescence-based microplate reader and a reaction volume of 100 µL per assay.

Acetylcholine, Acetylch linesterase and Choline Oxidase

Acetylcholine, the neurotransmitter released from the nerve terminal at neuromuscular junctions, binds to the acetylcholine receptor and opens its transmitter-gated ion channel (Section 16.3). The action of acetylcholine (ACh) is regulated by acetylcholinesterase (AChE), which hydrolyzes ACh to choline and acetate. The Amplex Red Acetylcholine/Acetylcholinesterase Assay Kit (A-12217) provides an ultrasensitive method for continuously monitoring AChE activity or for detecting ACh in a fluorescence-based microplate reader or a fluorometer. The kit can also be used for the assay of free choline, a newly declared "essential nutrient," in foods.[31] Potential uses for this kit include screening for AChE inhibitors and measuring the release of ACh from synaptosomes. In the assay, AChE activity is monitored indirectly using the Amplex Red reagent (see above). First, AChE converts the acetylcholine substrate to choline. Choline is in turn oxidized by choline oxidase to betaine and H_2O_2, the latter of which, in the presence of HRP, reacts with the Amplex Red reagent to generate the red-fluorescent product, resorufin (Figure 10.5). Experiments with purified AChE from electric eel indicate that the Amplex Red Acetylcholine/Acetylcholinesterase Assay Kit can detect AChE levels as low as 0.002 U/mL using a reaction time of one hour (Figure 16.28). We have been able to detect acetylcholinesterase activity from a tissue sample with total protein content as low as 200 ng/mL or 20 ng/well in a microplate assay.[32] By providing an excess of AChE in the assay, the kit can also be used to detect acetylcholine levels as low as 0.3 µM, with a range of detection from 0.3 µM to ~100 µM acetylcholine (Figure 16.29). Each kit contains:

- The Amplex Red reagent
- Horseradish peroxidase (HRP)
- H_2O_2 for use as a positive control
- DMSO and a concentrated reaction buffer
- Choline oxidase
- Acetylcholine (ACh)
- Acetylcholinesterase (AChE)
- A detailed protocol

Each kit provides a sufficient amount of each reagent for approximately 500 assays using a reaction volume of 200 µL per assay.

Xanthine and Xanthine Oxidase

The Amplex Red Xanthine/Xanthine Oxidase Assay Kit (A-22182) provides an ultrasensitive method for detecting xanthine or hypoxanthine or for monitoring xanthine oxidase activity. In the assay, xanthine oxidase catalyzes the oxidation of purine nucleotides, hypoxanthine or xanthine, to uric acid and superoxide. In the reaction mixture, the superoxide spontaneously degrades to H_2O_2, which in the presence of HRP reacts stoichiometrically with the Amplex Red reagent to generate the red-fluorescent oxidation product, resorufin. Resorufin has absorption and fluorescence emission maxima of approximately 563 nm and 587 nm, respectively, and because the extinction coefficient is high (54,000 $cm^{-1}M^{-1}$), the assay can be performed either fluorometrically or spectrophotometrically.

In healthy individuals, xanthine oxidase is present in appreciable amounts only in the liver and jejunum. However, in various liver disorders the enzyme is released into circulation. Therefore, determination of serum xanthine oxidase level serves as a sensitive indicator of acute liver damage such as jaundice. Previously, researchers have utilized chemiluminescence or absorbance to monitor xanthine oxidase activity. Using the Amplex Red Xanthine/Xanthine Oxidase Assay Kit, it is

possible to detect xanthine oxidase in a purified system at levels as low as 0.1 mU/mL by fluorescence (Figure 19.3). The kit can also be used to detect as little as 200 nM hypoxanthine or xanthine (Figure 19.4).

The Amplex Red Xanthine/Xanthine Oxidase Assay Kit (A-22182) contains:

- The Amplex Red reagent
- Horseradish peroxidase (HRP)
- H_2O_2
- DMSO and a concentrated reaction buffer
- Xanthine oxidase
- Xanthine
- Hypoxanthine
- A detailed protocol

Each kit provides sufficient reagents for approximately 400 assays using either a fluorescence- or absorbance-based microplate reader and reaction volumes of 100 µL per assay.

Uric Acid and Uricase

Serum uric acid is the end product of purine metabolism in the body tissues and is cleared through the kidneys by glomerular filtration. Most animals can metabolize uric acid to more readily excreted products, but humans lack the necessary enzyme, urate oxidase (uricase), as a result of the presence of two "nonsense mutations" in the human gene for uricase. Increased uric acid levels may result from leukemia, polycythemia, ingestion of foods high in nucleoproteins (e.g., liver and kidney) or impaired renal function. Gout results from the deposit of uric acid in body joints.

The Amplex Red Uric Acid/Uricase Assay Kit (A-22181) provides an ultrasensitive method for detecting uric acid or for monitoring uricase activity. In the assay, uricase catalyzes the conversion of uric acid to allantoin, H_2O_2 and carbon dioxide. In the presence of HRP the H_2O_2 reacts stoichiometrically with Amplex Red reagent to generate the red-fluorescent oxidation product, resorufin. Resorufin has absorption and fluorescence emission maxima of approximately 563 nm and 587 nm, respectively, and because the extinction coefficient is high (54,000 $cm^{-1}M^{-1}$), the assay can be performed either fluorometrically or spectrophotometrically. Previous literature reports colorimetric colorimetric detection limits at 3.6 µM, whereas the Amplex Red Uric Acid/Uricase Assay Kit can be used to detect as little as 100 nM uric acid in a purified system. The kit can also be used to detect a little as 0.2 mU/mL uricase in a purified system.

The Amplex Red Uric Acid/Uricase Assay Kit (A-22181) contains:

- The Amplex Red reagent
- Horseradish peroxidase (HRP)
- H_2O_2
- DMSO and a concentrated reaction buffer
- Uricase
- Uric acid
- A detailed protocol

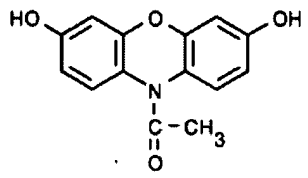
Each kit provides sufficient reagents for approximately 400 assays using either a fluorescence- or absorbance-based microplate reader and reaction volumes of 100 µL per assay.

1. Anal Biochem 253, 162 (1997); **2.** Anal Biochem 287, 196 (2000); **3.** J Immunol Methods 202, 133 (1997); **4.** Anal Biochem 253, 169 (1997); **5.** J Invest Dermatol 112, 751 (1999); **6.** Free Radic Biol Med 27, 1197 (1999); **7.** J Invest Dermatol 110, 966 (1998); **8.** Anal Chim Acta 402, 47 (1999); **9.** Anal Biochem 11, 6 (1965); **10.** J Clin Invest 87, 711 (1991); **11.** J Lab Clin Med 117, 291 (1991); **12.** Anal Biochem 187, 129 (1990); **13.** Anal Biochem 134, 111 (1983); **14.** Angew

Chem Int Ed Engl 39, 1453 (2000); **15.** Biochim Biophys Acta 1210, 151 (1994); **16.** Clin Chem 31, 1335 (1985); **17.** J Neurochem 39, 248 (1982); **18.** Nature 305, 158 (1983); **19.** Nucleic Acids Res 20, 5061 (1992); **20.** J Biolumin Chemilumin 9, 355 (1994); **21.** Cell Mol Neurobiol 18, 565 (1998); **22.** Methods Enzymol 233, 495 (1994); **23.** Anal Biochem 199, 191 (1991); **24.** J Biol Chem 275, 15749 (2000); **25.** Anal Biochem 284, 87 (2000); **26.** J Biochem Biophys Methods 38, 43 (1999); **27.** Methods Enzymol 142, 617 (1987); **28.** Anal Biochem 244, 384 (1997); **29.** Biochem Pharmacol 18, 1447 (1969); **30.** Anal Biochem 284, 382 (2000); **31.** Science 281, 794 (1998); **32.** Proc SPIE-Int Soc Opt Eng 3926, 166 (2000).

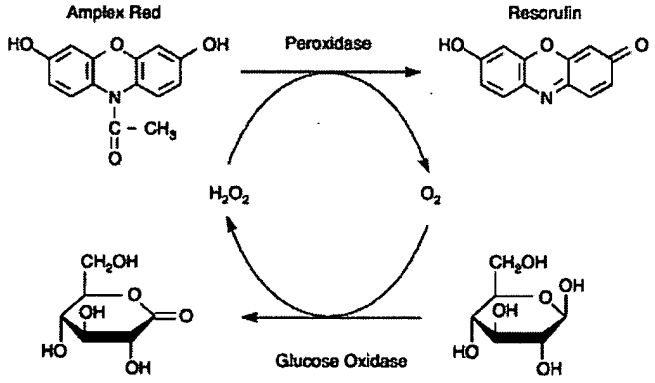
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Figure 10.49 A-12222 Amplex Red reagent.



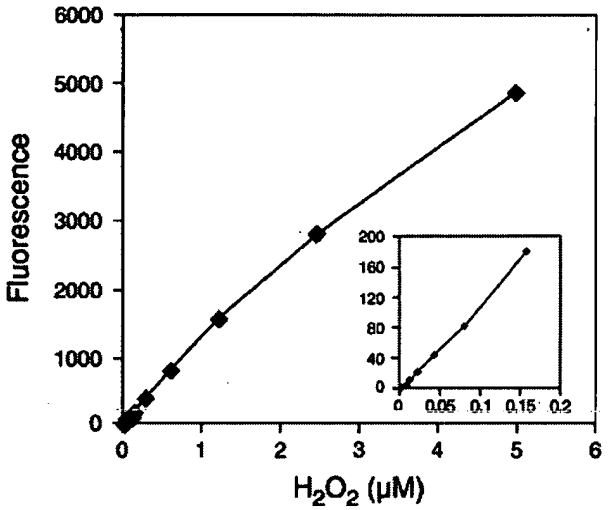
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Figure 10.50 Principle of coupled enzymatic assays using our Amplex Red reagent. Oxidation of glucose by glucose oxidase results in generation of H_2O_2, which is coupled to conversion of the Amplex Red reagent to fluorescent resorufin by HRP. The detection scheme shown here is used in our Amplex Red Glucose/Glucose Oxidase Assay Kit (A-22189).



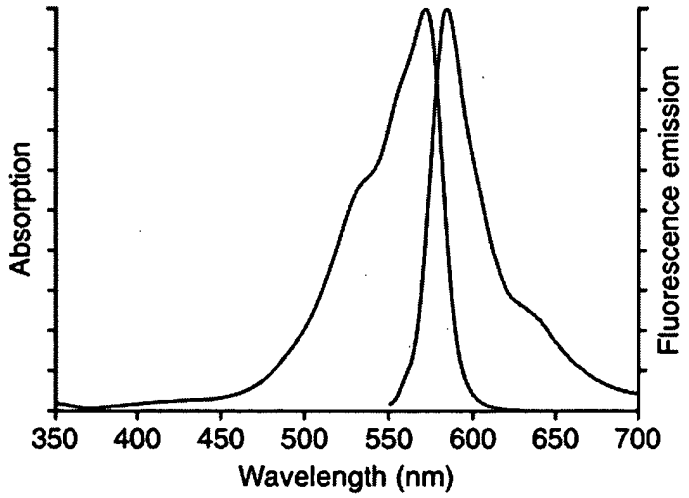
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Figure 10.51 Detection of H_2O_2 using the Amplex Red Hydrogen Peroxide/Peroxidase Assay Kit (A-22188). Reactions containing 50 µM Amplex Red reagent, 1 U/mL HRP and the indicated amount of H_2O_2 in 50 mM sodium phosphate buffer, pH 7.4, were incubated for 30 minutes at room temperature. Fluorescence was measured with a fluorescence-based microplate reader using excitation at 530 ± 12.5 nm and fluorescence detection at 580 ± 25 nm. Background fluorescence (24 units), determined for a no-H_2O_2 control reaction, was subtracted from each value. The inset shows the sensitivity and linearity of the assay at low levels of H_2O_2.



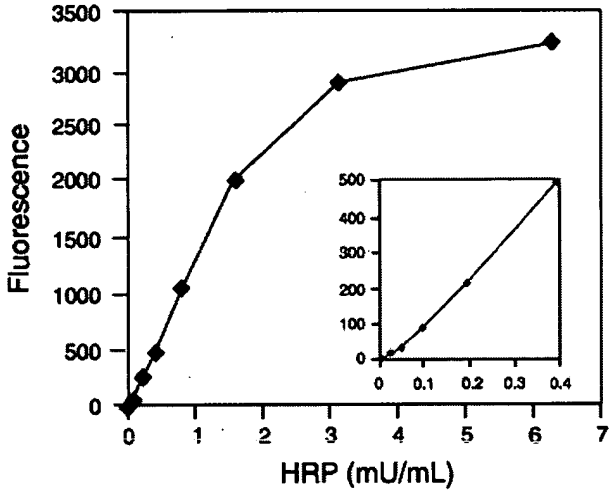
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Figure 10.52 Absorption and fluorescence emission spectra of resorufin (R-363) in pH 9.0 buffer.



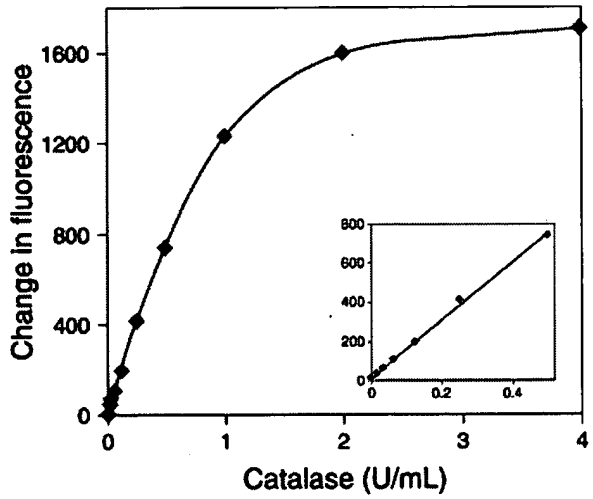
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Figure 10.53 Detection of HRP using the Amplex Red Hydrogen Peroxide/Peroxidase Assay Kit (A-22188). Reactions containing 50 µM Amplex Red reagent, 1 mM H_2O_2 and the indicated amount of HRP in 50 mM sodium phosphate buffer, pH 7.4, were incubated for 30 minutes at room temperature. Fluorescence was measured with a fluorescence-based microplate reader using excitation at 530 ± 12.5 nm and fluorescence detection at 590 ± 17.5 nm. Background fluorescence (3 units), determined for a no-HRP control reaction, was subtracted from each value. The inset shows the sensitivity of the assay at very low levels of HRP.



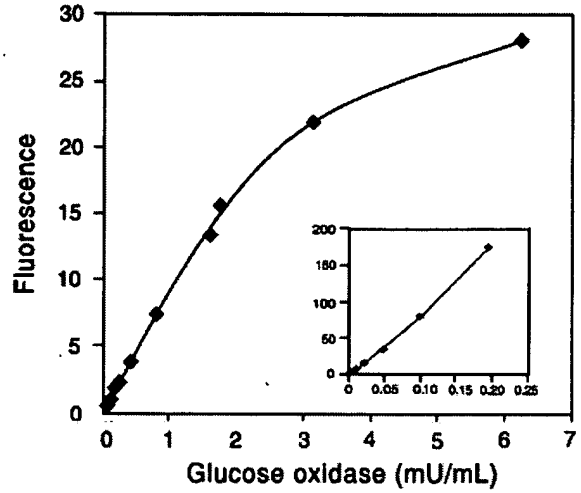
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Figure 10.54 Detection of catalase using the Amplex Red Catalase Assay Kit (A-22180). Reactions contained the indicated amount of catalase and 20 µM H_2O_2 in 1x Reaction Buffer and was incubated for 30 minutes. The final reaction containing 50 µM Amplex Red reagent and 0.2 U/mL HRP and was incubated at 37°C. After 30 minutes, fluorescence was measured in a fluorescence-based microplate reader using excitation at 530 ± 12.5 nm and fluorescence detection at 590 ± 17.5 nm. The change in fluorescence is reported as the observed fluorescence intensity subtracted from that of a no-catalase control.



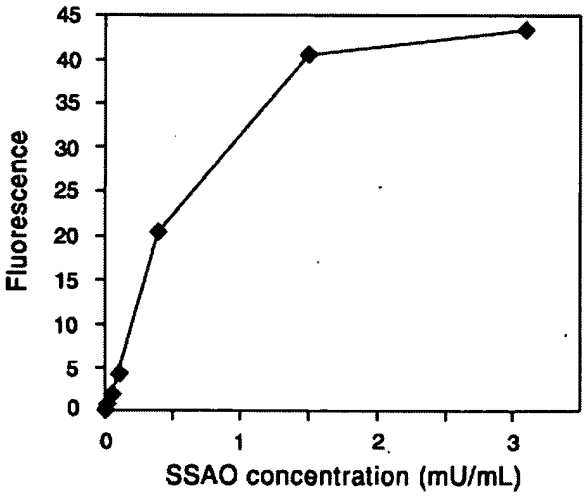
* * * * *

Figure 10.55 Detection of glucose oxidase using the Amplex Red Glucose/Glucose Oxidase Assay Kit (A-22189). Reactions containing 50 µM Amplex Red reagent, 1 U/mL HRP, 50 mM glucose and the indicated amount of glucose oxidase in 50 mM sodium phosphate buffer, pH 7.4, were incubated for 30 minutes at room temperature. Fluorescence was measured with a fluorescence-based microplate reader using excitation at 530 ± 12.5 nm and fluorescence detection at 590 ± 17.5 nm. Background fluorescence (19 units) determined for a no-glucose oxidase control reaction was subtracted from each value. The inset shows the assay's sensitivity at low levels of glucose oxidase (0–0.2 mU/mL).



* * * * *

Figure 10.56 Detection of plasma amine oxidase (an SSAO) activity using the Amplex Red Monoamine Oxidase Assay Kit (A-12214) and benzylamine as the substrate. Each reaction contained 1 mM benzylamine, 1 U/mL HRP, 200 µM Amplex Red reagent and the indicated amount of SSAO in 50 mM potassium phosphate, pH 7.4. Reactions were incubated at room temperature for 15 minutes. Fluorescence was measured with a fluorescence-based microplate reader using excitation at 560 ± 10 nm and fluorescence detection at 590 ± 10 nm.



* * * * *

Product List — 10.5 Substrates for Oxidases, Including Amplex Red Kits

Cat #	Pr duct Name	Unit Size

A-12217	Amplex® Red Acetylcholine/Acetylcholinesterase Assay Kit *500 assays*	1 kit
A-22180	Amplex® Red Catalase Assay Kit *400 assays*	1 kit
A-12216	Amplex® Red Cholesterol Assay Kit *500 assays*	1 kit
A-22170	Amplex® Red ELISA Kit #1 *with goat anti-mouse IgG, horseradish peroxidase conjugate* *1000 assays*	1 kit
A-22171	Amplex® Red ELISA Kit #2 *with protein G, horseradish peroxidase conjugate* *1000 assays*	1 kit
A-22179	Amplex® Red Galactose/Galactose Oxidase Assay Kit *400 assays*	1 kit
A-22189	Amplex® Red Glucose/Glucose Oxidase Assay Kit *500 assays*	1 kit
A-12221	Amplex® Red Glutamic Acid/Glutamate Oxidase Assay Kit *200 assays*	1 kit
A-22188	Amplex® Red Hydrogen Peroxide/Peroxidase Assay Kit *500 assays*	1 kit
A-12214	Amplex® Red Monoamine Oxidase Assay Kit *500 assays*	1 kit
A-22178	Amplex® Red Neuraminidase (Sialidase) Assay Kit *400 assays*	1 kit
A-12218	Amplex® Red Phosphatidylcholine-Specific Phospholipase C Assay Kit *500 assays*	1 kit
A-12219	Amplex® Red Phospholipase D Assay Kit *500 assays*	1 kit
A-12222	Amplex® Red reagent (10-acetyl-3,7-dihydroxyphenoxazine)	5 mg
A-22177	Amplex® Red reagent *packaged for high-throughput screening*	10 x 10 mg
A-12220	Amplex® Red Sphingomyelinase Assay Kit *500 assays*	1 kit
A-22181	Amplex® Red Uric Acid/Uricase Assay Kit *400 assays*	1 kit
A-22182	Amplex® Red Xanthine/Xanthine Oxidase Assay Kit *400 assays*	1 kit
C-400	5-(and-6)-carboxy-2',7'-dichlorodihydrofluorescein diacetate (carboxy-H_2DCFDA) *mixed isomers*	25 mg
C-13293	5-(and-6)-carboxy-2',7'-difluorodihydrofluorescein diacetate (carboxy-H_2DFFDA) *mixed isomers*	5 mg
D-22185	Diaminobenzidine (DAB) Histochemistry Kit #1 *with goat anti-mouse IgG–HRP*	1 kit
D-22186	Diaminobenzidine (DAB) Histochemistry Kit #2 *with goat anti-rabbit IgG–HRP*	1 kit
D-22187	Diaminobenzidine (DAB) Histochemistry Kit #3 *with streptavidin–HRP*	1 kit
D-399	2',7'-dichlorodihydrofluorescein diacetate (2',7'-dichlorofluorescin diacetate; H_2DCFDA)	100 mg
D-23805	dihydrocalcein, AM *special packaging*	20 x 50 µg
G-21040	goat anti-mouse IgG (H+L), horseradish peroxidase conjugate	1 mg
L-8455	luminol (3-aminophthalhydrazide)	25 g
M-20490	*N*-methyl-4-hydrazino-7-nitrobenzofurazan (NBD methylhydrazine)	25 mg
M-23800	2-methyl-6-(4-methoxyphenyl)-3,7-dihydroimidazo[1,2-a]pyrazin-3-one,	5 mg

	hydrochloride (MCLA)	
P-22061	P_iPer™ Phosphate Assay Kit *1000 assays*	1 kit
P-22062	P_iPer™ Pyrophosphate Assay Kit *1000 assays*	1 kit
P-21041	protein G, horseradish peroxidase conjugate	1 mg

* * * * *

Data Table — 10.5 Substrates for Oxidases, Including Amplex Red Kits

Cat #	MW	Storage	Soluble	Abs	EC	Em	Solvent	Product	Notes
A-6551	425.57	FF,D,AA	DMSO	286	8,000	none	MeOH	see Notes	1
A-12222	257.25	FF,D,A	DMSO	280	6,000	none	pH 8	R-363 (&item=363">see data)	2
A-22177	257.25	FF,D,A	DMSO	280	6,000	none	pH 8	R-363 (see data)	2
C-400	531.30	F,D	DMSO, EtOH	290	5,600	none	MeCN	see Notes	3
C-13293	498.39	F,D	DMSO, EtOH	290	5,500	none	MeCN	see Notes	4
D-399	487.29	F,D	DMSO, EtOH	258	11,000	none	MeOH	see Notes	3
D-1194	418.40	F,D,AA	DMSO, EtOH	272	4,600	none	MeOH	see Notes	3
D-23805	1068.95	F,D	DMSO	285	5,800	none	MeCN	C-481 (see data)	
L-8455	177.16	D,L	DMF	355	7,500	411	MeOH	see Notes	5
M-20490	209.16	F,L	MeCN	487	24,000	none	MeOH	see Notes	6
M-23800	291.74	FF,D,LL,AA	DMSO	430	8,400	546	MeOH	see Notes	7

Notes

1. Peroxidase-catalyzed reaction of A-6551 with H_2O_2 produces fluorescent 2-dodecylresorufin (Abs = 578 nm (EC = 69,000 $cm^{-1}M^{-1}$), Em = 597 nm in MeOH).
2. This substrate is used for peroxidase-coupled detection in our Amplex Red Assay Kits.
3. Dihydrofluorescein diacetates are colorless and nonfluorescent until both the acetates are hydrolyzed and the products are subsequently oxidized to fluorescein derivatives. The materials contain less than 0.1% of oxidized derivative when initially prepared. The end products from C-400, C-2938, C-6827, D-399 and D-2935 are 2',7'-dichlorofluorescein derivatives with spectra similar to C-368.
4. Difluorodihydrofluorescein diacetates are colorless and nonfluorescent. Acetate hydrolysis and subsequent oxidation generate a fluorescent 2',7'-difluorofluorescein derivative with spectra similar to O-6146.
5. This compound emits chemiluminescence upon oxidation in basic aqueous solutions. Emission peaks are at 425 nm (L-8455) and 470 nm (L-6868).
6. Peroxidase-catalyzed oxidation of NBD methyhydrazine generates fluorescent *N*-methyl-4-amino-7-nitrobenzofurazan, Abs = 470 nm, Em = 547 nm in aqueous buffer (pH 5.8) (Angew Chem Int Ed Engl 39, 1453 (2000)).

7. Generates chemiluminescence (Em = 455 nm) upon reaction with superoxide.

* * * * *

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Section 10.6 — Substrates for Miscellaneous Enzymes

Fluorogenic substrates that detect glycosidases (Section 10.2) and phosphatases (Section 10.3) have been by far the dominant probes for measuring enzymatic activity. Exactly the same fluorophores and chromophores — fluoresceins, resorufins and umbelliferones (7-hydroxycoumarins) — can be used to prepare substrates for other hydrolytic enzymes and ether-metabolizing microsomal dealkylase (cytochrome) enzymes or peroxidases. In addition, we offer substrates for chloramphenicol acetyltransferase (CAT) and luciferase, which are usually not widely expressed in cells. These substrates are important tools for detecting cells transfected with reporter genes that encode the enzymes. We also have available several reagents that are substrates for detecting enzyme-catalyzed chemical reduction associated with cells, including the tetrazolium salts MTT and XTT [1] (M-6494, X-6493; Section 15.2) and resazurin (R-12204), which under the name alamarBlue (a trademark of AccuMed International, Inc.) is useful for quantitatively measuring cell-mediated cytotoxicity,[2] cell proliferation [3,4] and mitochondrial metabolic activity in isolated neural tissue.[5]

Microsomal Dealkylases (Cytochrome P450)

Metabolic oxidation of chemical compounds, including many pollutants, is the function of the cytochrome-mediated monooxygenase or mixed-function oxidase system. Several enzymes are involved, including cytochrome P448 monooxygenase (aryl hydrocarbon hydroxylase), which is induced by carcinogenic polyaromatic hydrocarbons. Cytochrome P450 is a useful marker of endoplasmic reticulum membranes.[6] The very low turnover rate of these enzymes can be followed using various fluorogenic alkyl ether derivatives of coumarin,[7] resorufin [8] and fluorescein.[9]

Resorufin-Based Microsomal Dealkylase Substrates

The four resorufin ether substrates (R-351, R-352, R-441, R-1147), which all yield fluorescent resorufin (excitation/emission maxima ~571/585 nm, Figure 10.5), have been extensively used to differentiate isozymes of cytochrome P450.[8,10–16] A fluorescence microplate based–assay has been developed for the simultaneous determination of ethoxyresorufin-*O*-deethylase and total protein concentration using resorufin ethyl ether (ethoxyresorufin, R-352) and fluorescamine [17] (F-2332; FluoroPure Grade, F-20261; Section 9.2).

Coumarin-Based Microsomal Dealkylase Substrates

Fluorescence detection of the deethylation of 3-cyano-7-ethoxycoumarin [10] (C-684) is reported to be 50–100 times more sensitive than that of ethoxyresorufin, primarily because of the faster turnover rate of 3-cyano-7-ethoxycoumarin;[18,19] however, ethoxyresorufin exhibits lower fluorescence background due to its more favorable spectral shifts. The deethylase product of 3-cyano-7-ethoxycoumarin, 3-cyano-7-hydroxycoumarin (C-183, Section 10.1), has a lower pK_a than that of 7-ethoxycoumarin,[20,21] allowing continuous measurements of enzyme activity at pH 7.

The cytochrome P-450 substrate 7-ethoxy-4-trifluoromethylcoumarin (E-2882) yields a product with a fluorescence emission that is distinct from that of the substrate and of NADPH, making this substrate useful for the direct measurement of enzymatic activity.[22–25] Researchers have shown that this substrate is cleaved by at least the 1A2, 2E1 and 2B1 isozymes of cytochrome P450.[23,26]

Microsomal Dealkylase Substrates

The fluorescent products of most microsomal dealkylase substrates rapidly leak from live cells, making them ineffective for measuring intracellular enzymatic activity by imaging or flow cytometric analysis. Using techniques for product retention that proved successful for our patented DetectaGene and ImaGene glycosidase substrates, we have developed unique substrates that can potentially be used to detect dealkylase activity in single cells.

Like the DetectaGene β-galactosidase substrates, the mildly thiol-reactive chloromethyl moiety in our 5-chloromethylfluorescein diethyl ether (C-6533) reacts with glutathione or other intracellular thiols to produce a product that is retained in cells through cell division. Adding a chloromethyl moiety to our glycosidase and peptidase substrates (Section 10.2, Section 10.4; Figure 10.16) has enabled researchers to identify cells with enzymatic activity 24 hours after loading the substrate.[27] Fluorescein ethers are known microsomal dealkylase substrates.[19] Chloromethylfluorescein diethyl ether was used to monitor cytochrome P450–dependent mixed function oxidase activity in cultured hepatocytes.[28] The low pK_a (pK_a ~4.9) of the hydrolysis product of CMDiFUEt, 6,8-difluoro-7-hydroxycoumarin (DiFMU, D-6566; Section 10.1; Figure 10.29), permits the assay of microsomal dealkylase activity at pH greater than or equal to 6.[29]

Lipases

Lipases generally include cholesterol esterases and glycerol ester hydrolases. Phospholipase A selectively hydrolyzes lipophilic esters of phospholipids. Because of their importance to the process of signal transduction in cells, our extensive selection of substrates and other probes for phospholipases is discussed in Section 18.4. The cholesterol produced by cholesterol esterases is readily quantitated using the Amplex Red Cholesterol Assay Kit (A-12216), which is discussed in Section 10.5 and Section 13.3. It should be possible to modify the Amplex Red assay for cholesterol to continuously measure the activity of cholesterol esterases. Unlike lipase substrates that are esters of 7-hydroxy-4-methylcoumarin (β-methylumbelliferone, H-189; Section 10.1) — a dye that is not appreciably fluorescent at neutral pH — 6,8-difluoro-4-methylumbelliferyl octanoate (DiFMU octanoate, D-12200) can be used for the continuous *in vitro* assay of lipases at pH greater than or equal to 6; the blue-fluorescent hydrolysis product of DiFMU octanoate, 6,8-difluoro-7-hydroxy-4-methylcoumarin (DiFMU, D-6566; Section 10.1; Figure 10.29), has a pK_a of 4.9.[29]

Acetylcholinesterase and Histone Acetyltransferase

The Amplex Red Acetylcholine/Acetylcholinesterase Assay Kit (A-12217, Section 10.5, Section 16.2) provides an ultrasensitive method for continuously monitoring acetylcholinesterase activity or for detecting acetylcholine in a fluorescence-based microplate reader or fluorometer (Figure 16.28, Figure 16.29).

Acetylcholinesterase has also been assayed using the acetylcholinesterase-catalyzed release of thiocholine from acetylthiocholine, followed by detection of the thiol with the essentially nonfluorescent 7-diethylamino-3-(4'-maleimidylphenyl)-4-methylcoumarin [30] (CPM, D-346; Section 2.3) dye or Ellman's reagent [31] (DTNB, D-8451; Section 2.3).

In a similar assay, the activity of histone acetyltransferase, which transfers an acetyl group from acetyl coenzyme A to lysine residues in the N-terminal tail of histones, was measured by using CPM to derivatize the thiol-containing coenzyme A (CoASH) product.[32]

Chloramphenicol Acetyltransferase (CAT)

Because of the close correlation between its transcript levels and enzymatic activity and the excellent sensitivity of the enzyme assay, the chloramphenicol acetyltransferase (CAT) reporter gene system has proven to be a powerful tool for investigating transcriptional elements in animal

[33,34] and plant cells.[35,36] Most conventional CAT assays require incubation of cell extracts with radioactive substrates, typically ^{14}C chloramphenicol or ^{14}C acetyl CoA, followed by organic extraction and autoradiography or scintillation counting.[37–39] Molecular Probes' patented *FAST* CAT Chloramphenicol Acetyltransferase Assay Kits [40] contain unique BODIPY chloramphenicol fluorescent substrates that take advantage of the exquisite sensitivity of fluorescence techniques, thus eliminating the need for hazardous radiochemicals, film, fluors, scintillation counters and expensive radioactive waste disposal.[41,42] The original *FAST* CAT Kit and our *FAST* CAT Green and Yellow (deoxy) Kits provide detection limits similar to those achieved with conventional radioactive methods and yield results that are easily visualized using a hand-held UV lamp.

FAST CAT Chloramphenicol Acetyltransferase Assay Kit

The green-fluorescent BODIPY FL chloramphenicol substrate in our original *FAST* CAT Chloramphenicol Acetyltransferase Assay Kit (F-2900) has a K_M for purified CAT of 7.4 µM and a V_{max} of 375 pmole/unit/min, values that are similar to those of ^{14}C-labeled chloramphenicol [42,43] (K_M = 12 µM and V_{max} = 120 pmole/unit/min). To perform the assay, cell extracts are simply incubated with BODIPY FL chloramphenicol and acetyl coenzyme A. After a suitable incubation period, the products and remaining substrate are extracted and separated by thin-layer chromatography (TLC). The brightly fluorescent, well-resolved spots can be immediately visualized with a hand-held UV lamp or quantitated with a laser-based scanner or CCD camera (Figure 10.57). Alternatively, quantitation can be accomplished using a fluorometer or spectrophotometer after a simple extraction. HPLC analysis of the fluorescent products has also been used to further enhance the assay's sensitivity.[44,45]

These attributes have enabled researchers to use this *FAST* CAT substrate to measure CAT activity in crude cellular extracts of transfected ovarian granulosa cells.[43] Our *FAST* CAT Kit has also been employed to study hormonal regulation of prodynorphin gene expression [46,47] and to measure the rate of hair growth in single follicles of transgenic mice.[45]

Each *FAST* CAT Chloramphenicol Acetyltransferase Assay Kit (F-2900) is available in a 100-test size and includes:

- The BODIPY FL chloramphenicol substrate
- A mixture of the 1- and 3-acetyl and 1,3-diacetyl BODIPY FL derivatives, which serve as a reference standard for the fluorescent products
- A detailed protocol

FAST CAT (Deoxy) Chloramphenicol Acetyltransferase Assay Kits

Molecular Probes' two *FAST* CAT (deoxy) Chloramphenicol Acetyltransferase Assay Kits (F-6616, F-6617) contain substrates that greatly simplify the quantitation of chloramphenicol acetyltransferase (CAT) activity and extend the linear detection range of Molecular Probes' original *FAST* CAT assay.[48] The BODIPY FL chloramphenicol substrate in our original *FAST* CAT kit contains two acetylation sites, only one of which is acetylated by the CAT enzyme. Once the CAT enzyme adds an acetyl group to this position, the acetyl group is nonenzymatically transferred to the second site, leaving the original position open for another enzymatic acetylation.[49–51] Therefore, enzyme acetylation of our original BODIPY FL *FAST* CAT substrate produces three products — one diacetylated and two monoacetylated chloramphenicols — thus complicating the quantitative analysis of CAT gene activity. More importantly, because the nonenzymatic transacetylation is the rate-limiting step, the rate of product accumulation may not accurately reflect CAT activity.[49,52,53]

To overcome this limitation, we have modified the original *FAST* CAT substrate, producing reagents that undergo a single acetylation reaction (Figure 10.58). The green-fluorescent BODIPY FL

*deoxy*chloramphenicol and yellow-fluorescent BODIPY 543/569 *deoxy*chloramphenicol substrates in our *FAST* CAT Green and *FAST* CAT Yellow (deoxy) Chloramphenicol Acetyltransferase Assay Kits (F-6616, F-6617) are acetylated at a single position, yielding only one fluorescent product [48,53] (Figure 10.58). This simplified reaction scheme provides a straightforward and reliable measure of CAT activity and extends the linear detection range of our original *FAST* CAT assay.

The *FAST* CAT Green and *FAST* CAT Yellow (deoxy) Chloramphenicol Acetyltransferase Assay Kits are available in a 100-test size and include:

- The BODIPY FL 1-deoxychloramphenicol substrate (in Kit F-6616) or BODIPY TMR 1-deoxychloramphenicol substrate (in Kit F-6617)
- A 3-Acetyl BODIPY FL derivative (in Kit F-6616) or BODIPY TMR derivative (in Kit F-6617), which serves as a reference standard for the fluorescent product
- A detailed protocol

The CAT substrate in our *FAST* CAT Green (deoxy) Chloramphenicol Acetyltransferase Assay Kit (F-6616) is spectrally identical to the green-fluorescent BODIPY FL chloramphenicol substrate in our original *FAST* CAT Kit. The *FAST* CAT Yellow (deoxy) Chloramphenicol Acetyltransferase Assay Kit (F-6617) contains a red-orange–fluorescent BODIPY TMR derivative. The availability of two spectrally distinct CAT substrates allows researchers to choose the optimal fluorophore for a particular excitation source or multicolor labeling experiment.

Luciferase

Firefly luciferase (*Photinus*-luciferin:oxygen 4-oxidoreductase or luciferin 4-monooxygenase, EC 1.13.12.7) produces light by the ATP-dependent oxidation of luciferin (Figure 15.52). The 560 nm chemiluminescence from this reaction peaks within seconds, with light output that is proportional to luciferase concentration when substrates are present in excess.[54] The *luc* gene, which encodes the 62,000-dalton firefly luciferase, is a popular reporter gene for plants,[35,36,55–58] bacteria [59,60] and mammalian cells [61,62] and for monitoring baculovirus gene expression in insects.[63,64] Chemiluminescent techniques are virtually background-free, making the *luc* reporter gene ideal for detecting low-level gene expression.[65]

Luciferin

The substrate for firefly luciferase, D-(-)-2-(6'-hydroxy-2'-benzothiazolyl)thiazoline-4-carboxylic acid, commonly known as luciferin, was first isolated by Bitler and McElroy (9 mg from approximately 15,000 fireflies!).[66] In the firefly, spent luciferin (oxyluciferin) is recycled back to luciferin.[67] Molecular Probes is a primary manufacturer of synthetic luciferin (L-2911) and its water-soluble sodium (L-2912; FluoroPure Grade, L-22172) and potassium (L-2916) salts. The physical properties of these derivatives are identical to those of the natural compound. Our prices for luciferin are significantly lower than those of other suppliers; additional discounts are available for bulk purchases or standing orders.

Typically, luciferase expression is measured by adding the substrates ATP and luciferin to cell lysates and then analyzing light production with a luminometer. As little as 0.02 pg (250,000 molecules) of luciferase can be reliably measured using a standard scintillation counter.[68] Moreover, a CCD-imaging method of detecting *luc* gene expression in single cells has been developed.[69]

Caged Luciferin

Although luciferase activity is sometimes measured in living cells,[69,70] *in vivo* quantitation appears to be limited by the difficulty in delivering luciferin into intact cells.[71] Molecular Probes'

DMNPE-caged luciferin (L-7085) readily crosses cell membranes. Once the caged luciferin is inside the cell, active luciferin can be released either instantaneously by a flash of UV light or continuously by the action of endogenous intracellular esterases, which are found in many cell types. This probe should facilitate *in vivo* luciferase assays in two important ways. First, caged luciferin improves the sensitivity and quantitative analysis of these assays by allowing more efficient delivery of luciferin into intact cells. Second, hydrolysis by intracellular esterases provides a continuous supply of active luciferin, permitting long-term measurements and reducing the need for rapid mixing protocols and costly injection devices. Moreover, DMNPE-caged luciferin may make it easier to follow dynamic changes in gene expression in live cells. Molecular Probes also offers DMNPE-caged ATP (A-1049, Section 17.2), which can be used in conjunction with DMNPE-caged luciferin (L-7085) for this *in vivo* luciferase assay.[72]

Luciferin–Luciferase Assays for ATP, Anesthetics and Hormones

Luciferin has been used in an exquisitely sensitive and specific ATP assay,[73,74] which allows the detection of femtomolar quantities of ATP.[75] This bioluminescent ATP assay has been employed to determine cell proliferation and cytotoxicity in both bacteria [76,77] and mammalian cells.[78,79] Molecular Probes provides all the reagents needed for this important assay in its ATP Determination Kit (A-22066, Section 10.3).

Researchers have also adapted the luciferin–luciferase ATP assay system for detecting single base changes in a solid-phase DNA sequencing method.[80] In addition, amphipathic and hydrophobic substances, including certain anesthetics and hormones, compete with luciferin for the hydrophobic site on the luciferase molecule, providing a convenient method to assay subnanomolar concentrations of these substances.[81] A protein A–luciferase fusion protein has been developed that can be used in bioluminescence-based immunoassays.[82,83]

Resazurin Derivatives for Dehydrogenase Assays

Resazurin (R-12204), which under the name alamarBlue (a trademark of AccuMed International, Inc.) has been reported to be useful for quantitatively measuring cell-mediated cytotoxicity,[2] cell proliferation [3,4] and mitochondrial metabolic activity in isolated neural tissue, is also a useful substrate for measuring the dehydrogenase activity or a wide variety of dehydrogenase enzymes *in vitro*. Among the assays reported are the use of resazurin to detect:

- Serum formate using formate dehydrogenase and NAD^+ [84]
- Bile acids in human urine, feces and serum using NAD^+ 3α-hydroxysteroid dehydrogenase [85–89]
- Glucose 6-phosphate dehydrogenase (G6PD) activity [90]
- NADH and bile acids with NADH oxidoreductase [91,92]
- Triacylglycerols with glycerol dehydrogenase [93]
- Arginosuccinate lyase and NAPDH by a coupled diaphorase–resazurin reaction sequence [94]
- Urinary acylcarnitines in an immobilized enzyme reactor [95,96]

The dehydrogenase substrate in our Vybrant Cell Metabolic Assay Kit (V-23110, Section 15.3) is dodecylresazurin, a more lipophilic version of resazurin. Because this substrate readily penetrates the membranes of live cells and its fluorescent reduction product (dodecylresorufin) is better retained in cells, it is preferred for both cell-based or flow cytometric assays. See Section 15.3 for more details.

Glucose 6-Phosphate Dehydrogenase (G6PD) Assay

Glucose 6-phosphate dehydrogenase (G6PD) is a ubiquitous enzyme that is part of the pentose

phosphate pathway, and is crucial for cellular antioxidant defenses via its production of NADPH.[97,98] Our Vybrant Cytotoxicity Assay Kit (V-23111) was designed to monitor the release of this cytosolic enzyme from damaged cells into the surrounding medium. However, our method also provides an extremely sensitive and specific assay for G6PD in cell-free extracts. Detection of G6PD is via a two-step enzymatic process that leads to the reduction of resazurin into the red-fluorescent resorufin (Figure 15.34). The resulting signal is proportional to the amount of G6PD released into the cell media, which correlates with the number of dead cells in the sample (Figure 15.35).

The Vybrant Cytotoxicity Assay Kit contains all enzymes and substrates needed to measure the activity of G6PD and to detect the release of G6PD from damaged and dying cells. The assay can be completed in less than an hour and is effective with as few as 500 cells per sample. Resorufin, the end product of the G6PD cytotoxicity assay, has absorption and emission maxima at 563 nm and 585 nm, respectively, placing the fluorescent signal beyond the autofluorescence of most biological samples. In addition, the levels of G6PD in serum commonly used for cell culture are lower than those of lactate dehydrogenase (LDH), an enzyme often used in similar assays, thus resulting in lower background signals (Figure 15.36). The Vybrant Cytotoxicity Assay Kit (V-23111) contains:

- Resazurin (5 vials)
- DMSO
- Reaction mixture (diaphorase, glucose 6-phosphate and $NADP^+$)
- Reaction buffer
- Cell lysis buffer
- A detailed protocol for the assay

Sufficient reagents are provided for about 1000 assays in a fluorescence-based microplate reader.

Nitroreductase/Nitrate Reductase

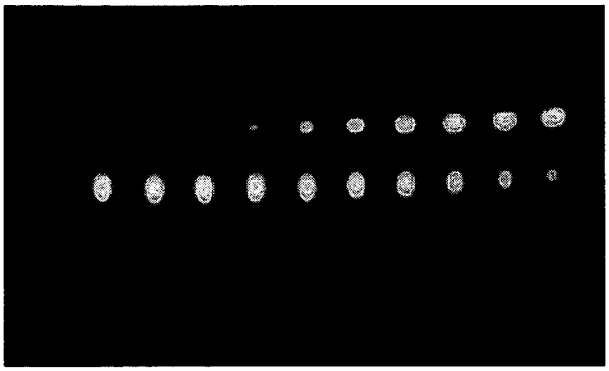
We have developed a unique fluorogenic substrate that can detect the enzymatic activity of certain enzymes that reduce nitro compounds to amines or inorganic nitrate to nitrite. 6-Chloro-9-nitro-5-oxo-5*H*-benzo[a]phenoxazine (CNOB, C-22220; Figure 10.59) is reduced to an aminophenoxazine dye that absorbs maximally at ~620 nm and has an emission maximum near 630 nm. We have shown that CNOB is a good substrate for at least some bacterial nitroreductases but apparently is not a good substrate for a mammalian nitroreductase. The utility of CNOB for detection of nitroreductase activity or detection of hypoxia in tumor cells has not yet been tested; however, it is known that some nitroimidazoles and other nitroaromatic compounds are reduced to amines under highly reducing conditions.[99]

1. J Immunol Methods 142, 257 (1991); **2.** J Immunol Methods 213, 157 (1998); **3.** J Clin Lab Anal 9, 89 (1995); **4.** J Immunol Methods 175, 181 (1994); **5.** J Neurosci Res 45, 216 (1996); **6.** J Biol Chem 270, 24327 (1995); **7.** Anal Biochem 191, 354 (1990); **8.** Biochem J 240, 27 (1986); **9.** Anal Biochem 133, 46 (1983); **10.** Anal Biochem 248, 188 (1997); **11.** Biochem Pharmacol 47, 893 (1994); **12.** Biochem Pharmacol 46, 933 (1993); **13.** Anal Biochem 188, 317 (1990); **14.** Biochem Pharmacol 40, 2145 (1990); **15.** Eur J Immunol 16, 829 (1986); **16.** Biochem Pharmacol 34, 3337 (1985); **17.** Anal Biochem 222, 217 (1994); **18.** Anal Biochem 172, 304 (1988); **19.** Biochem J 247, 23 (1987); **20.** Anal Biochem 115, 177 (1981); **21.** Hoppe Seylers Z Physiol Chem 353, 1171 (1972); **22.** Biochemistry 37, 13184 (1998); **23.** Biochem Pharmacol 46, 1577 (1993); **24.** Biochem Pharmacol 37, 1731 (1988); **25.** Biochemistry 36, 11707 (1997); **26.** Arch Biochem Biophys 323, 303 (1995); **27.** J Neurosci 15, 1025 (1995); **28.** Int J Artif Organs 21, 360 (1998); **29.** Bioorg Med Chem Lett 8, 3107 (1998); **30.** Anal Biochem 133, 450 (1983); **31.** Biochem Pharmacol 7, 88 (1961); **32.** Anal Biochem 287, 319 (2000); **33.** Development 109, 577 (1990); **34.** Proc Natl Acad Sci U S A 87, 6848 (1990); **35.** Methods Cell Biol 50, 425 (1995); **36.** Methods Mol Biol 55, 147 (1995); **37.** Gene 67, 271 (1988); **38.** J Virol 62, 297 (1988); **39.** Anal Biochem 156, 251 (1986); **40.** US 5,262,545 and US 5,364,764; **41.** Methods Enzymol 216, 369 (1992); **42.** Biotechniques 8, 170 (1990); **43.** Anal Biochem 197, 401 (1991); **44.** J Biol Chem 270, 28392 (1995); **45.** Eur J Clin Chem Clin Biochem 31, 41 (1993); **46.** Mol Cell Neurosci 3, 278 (1992); **47.**

Mol Endocrinol 6, 2244 (1992); **48.** Biotechniques 19, 488 (1995); **49.** Biochemistry 30, 3763 (1991); **50.** Biochemistry 30, 3758 (1991); **51.** Biochemistry 29, 2075 (1990); **52.** Annu Rev Biophys Biophys Chem 20, 363 (1991); **53.** Nucleic Acids Res 19, 6648 (1991); **54.** Mol Cell Biol 7, 725 (1987); **55.** J Biolumin Chemilumin 8, 267 (1993); **56.** Methods Enzymol 216, 397 (1992); **57.** Dev Genet 11, 224 (1990); **58.** J Biolumin Chemilumin 5, 141 (1990); **59.** J Gen Microbiol 138, 1289 (1992); **60.** Methods Mol Cell Biol 1, 107 (1989); **61.** Methods Mol Biol 7, 237 (1991); **62.** Biotechniques 7, 1116 (1989); **63.** FEBS Lett 274, 23 (1990); **64.** Gene 91, 135 (1990); **65.** Anal Biochem 176, 28 (1989); **66.** Arch Biochem Biophys 72, 358 (1957); **67.** J Biol Chem 276, 36508 (2001); **68.** Anal Biochem 171, 404 (1988); **69.** J Biolumin Chemilumin 5, 123 (1990); **70.** Biotechnology (N Y) 10, 565 (1992); **71.** Biochem J 276, 637 (1991); **72.** Biotechniques 15, 848 (1993); **73.** J Appl Biochem 3, 473 (1981); **74.** Anal Biochem 29, 381 (1969); **75.** Lett Appl Microbiol 1, 208 (1990); **76.** Biotechnol Bioeng 42, 30 (1993); **77.** J Biolumin Chemilumin 6, 193 (1991); **78.** Biochem J 295, 165 (1993); **79.** J Immunol Methods 160, 81 (1993); **80.** Anal Biochem 208, 171 (1993); **81.** Anal Biochem 190, 304 (1990); **82.** Anal Biochem 208, 300 (1993); **83.** J Immunol Methods 137, 199 (1991); **84.** J Anal Toxicol 8, 273 (1984); **85.** Clin Chem 29, 171 (1983); **86.** Arch Biochem Biophys 337, 121 (1997); **87.** J Membr Biol 159, 197 (1997); **88.** Clin Chim Acta 70, 79 (1976); **89.** Clin Chem 24, 1150 (1978); **90.** Biochim Biophys Acta 484, 249 (1977); **91.** Clin Chim Acta 107, 149 (1980); **92.** Clin Chim Acta 102, 241 (1980); **93.** Clin Chem 26, 613 (1980); **94.** Anal Biochem 164, 482 (1987); **95.** Clin Chim Acta 216, 135 (1993); **96.** Clin Chem 36, 2072 (1990); **97.** J Biol Chem 275, 40042 (2000); **98.** Nucleic Acids Res 14, 2511 (1986); **99.** Anticancer Drug Des 13, 687 (1998).

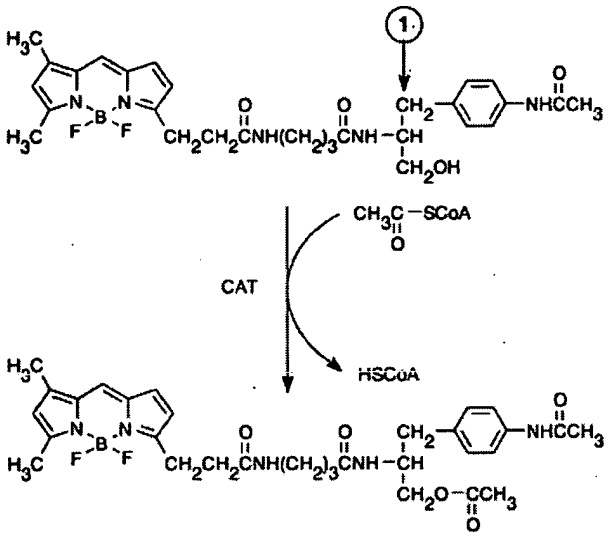
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Figure 10.57 Chloramphenicol acetyltransferase (CAT) assays using our *FAST* CAT Yellow (deoxy) Chloramphenicol Acetyltransferase Assay Kit (F-6617). Decreasing amounts of purified CAT enzyme (twofold dilutions) were incubated with the corresponding deoxy substrate in the presence of acetyl CoA; the reaction mixture was then separated with standard thin-layer chromatography (TLC) methods and visualized with 366 nm epi-illumination. The bottom row of fluorescent spots in each TLC represents the substrate; the top, the monoacetylated reaction product.



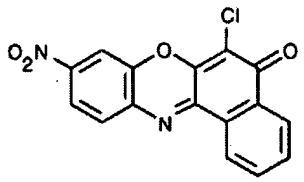
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Figure 10.58 The green-fluorescent BODIPY FL 1-deoxychloramphenicol substrate in our *FAST* CAT Green (deoxy) Chloramphenicol Acetyltransferase Assay Kit (F-6616). CAT-mediated acetylation of this substrate and of the BODIPY TMR 1-deoxychloramphenicol in our *FAST* CAT Yellow (deoxy) Chloramphenicol Acetyltransferase Assay Kit (F-6617) results in single fluorescent products because these substrates contain only one hydroxyl group that can be acetylated. In contrast, the BODIPY FL chloramphenicol substrate in our original *FAST* CAT Kit (F-2900) contains a second hydroxyl group at the 1-position (indicated by the labeled arrow). This hydroxyl group undergoes a nonenzymatic transacetylation step, restoring the original hydroxyl for a second acetylation. CAT-mediated acetylation of this chloramphenicol substrate produces three fluorescent products, thus complicating the analysis.



* * * * *

Figure 10.59 C-22220 6-chloro-9-nitro-5-oxo-5*H*-benzo[a]phenoxazine.



* * * * *

Product List — 10.6 Substrates for Miscellaneous Enzymes

Cat #	Pr duct Name	Unit Size
C-6533	5-(and-6)-chloromethylfluorescein diethyl ether *mixed isomers*	5 mg
C-22220	6-chloro-9-nitro-5-oxo-5*H*-benzo[a]phenoxazine (CNOB)	1 mg
C-684	3-cyano-7-ethoxycoumarin	10 mg
D-12200	6,8-difluoro-4-methylumbelliferyl octanoate (DiFMU octanoate)	10 mg
E-2882	7-ethoxy-4-trifluoromethylcoumarin	25 mg
F-2900	*FAST* CAT® Chloramphenicol Acetyltransferase Assay Kit *100 assays*	1 kit
F-6616	*FAST* CAT® Green (deoxy) Chloramphenicol Acetyltransferase Assay Kit *100 assays*	1 kit
F-6617	*FAST* CAT® Yellow (deoxy) Chloramphenicol Acetyltransferase Assay Kit *100 assays*	1 kit
L-7085	D-luciferin, 1-(4,5-dimethoxy-2-nitrophenyl)ethyl ester (DMNPE-caged luciferin)	5 mg
L-2911	D-luciferin, free acid	25 mg
L-2916	D-luciferin, potassium salt	25 mg
L-2912	D-luciferin, sodium salt	25 mg
L-22172	D-luciferin, sodium salt *FluoroPure™ grade*	10 mg
R-12204	resazurin, sodium salt	10 mg
R-441	resorufin benzyl ether (benzyloxyresorufin)	10 mg
R-352	resorufin ethyl ether (ethoxyresorufin)	5 mg
R-351	resorufin methyl ether (methoxyresorufin)	5 mg
R-1147	resorufin pentyl ether (pentoxyresorufin)	5 mg
V-23111	Vybrant® Cytotoxicity Assay Kit *G6PD release assay* *1000 assays*	1 kit

* * * * *

Data Table — 10.6 Substrates for Miscellaneous Enzymes

Cat #	MW	Storage	Soluble	Abs	EC	Em	Solvent	Product	Notes
A-6611	279.33	F,D,L,A	pH >6, DMSO	349	17,000	521	pH 7	see Notes	1
B-12270	579.42	F,D,L	DMSO	560	74,000	568	MeOH		
C-684	215.21	L	DMSO	356	20,000	411	pH 7	C-183 (&item=183">see data)	
C-6533	436.89	F,D	DMSO	275	8,200	none	MeOH	see Notes	2
C-7933	287.23	F,D,L	DMSO	299	16,000	431	MeOH	see Notes	3
C-12202	274.65	F,D	MeCN	288	8,600	none	MeOH	C-12881 (&item=12881">see data)	

C-22220	326.70	F,D,L	DMSO	448	13,000	none	MeOH	see Notes	4
D-12200	338.35	F,D	MeCN	312	5,000	none	MeCN	D-6566 (&item=6566">see data)	
D-12203	240.21	D	MeCN	315	8,500	none	MeCN	D-6566 (&item=6566">see data)	
E-2882	258.20	L	DMSO, DMF	333	14,000	415	MeOH	T-659 (&item=659">see data)	
E-6580	349.17	F,D,L	DMSO, DMF	289	16,000	ND	MeOH	E-6578 (&item=6578">see data)	5, 6
E-6583	545.55	F,D,L	DMSO, DMF	288	14,000	ND	MeOH	E-6578 (see data)	5, 6
F-2900	583.44	F,D,L	MeOH	504	80,000	511	MeOH	see Notes	7, 8
F-6489	529.94	F,D,L	DMF	452	15,000	519	pH 7	F-1300 (see data)	
F-6616	567.44	F,D,L	MeOH	504	81,000	510	MeOH	see Notes	8, 9
F-6617	673.57	F,D,L	MeOH	545	60,000	570	MeOH	see Notes	8, 9
G-7702	903.08	FF,D,L	$CHCl_3$	341	107,000	473	MeOH	P-1903 (&item=1903">see data)	
L-2911	280.32	F,D,L,A	pH >6, DMSO	328	18,000	532	pH 7	see Notes	10
L-2912	302.30	F,D,L,A	pH >6	328	17,000	533	pH 7	see Notes	10
L-2916	318.41	F,D,L,A	pH >6	328	18,000	533	pH 7	see Notes	10
L-7085	489.52	FF,D,LL	DMSO, DMF	334	22,000	none	MeOH	see Notes	11, 12
L-22172	302.30	F,D,L,A	pH >6	328	17,000	533	pH 7	see Notes	10, 13
R-351	227.22	L	DMSO	463	23,000	none	MeOH	R-363 (&item=363">see data)	
R-352	241.25	L	DMSO	464	23,000	none	MeOH	R-363 (&item=363">see data)	
R-441	303.32	L	DMSO	463	21,000	none	MeOH	R-363 (&item=363">see data)	
R-1147	283.33	L	DMSO	465	21,000	none	MeOH	R-363 (&item=363">see data)	
R-12204	251.17	L	H_2O, MeOH	604	60,000	none	MeOH	R-363 (&item=363">see data)	

Notes

1. ATP-dependent oxidation of A-6611 by luciferase results in bioluminescence (Em = 605 nm). The bioluminescence spectrum is pH-independent (J Am Chem Soc 88, 2015 (1966)).
2. Enzymatic cleavage of this substrate yields 5-chloromethylfluorescein, with spectroscopic properties similar to C-1904.
3. Enzymatic hydroxylation yields a 7-hydroxycoumarin derivative with spectroscopic properties similar to H-185.
4. Enzymatic reduction of C-22220 yields a fluorescent aminobenzophenoxazine derivative (Abs = 617 nm, Em = 625 nm).
5. ND = not determined.
6. Fluorescence of the unhydrolyzed substrate is very weak.
7. Acetylation by chloramphenicol acetyltransferase (CAT) yields a mixture of 1-acetyl, 3-acetyl and 1,3-diacetyl chloramphenicol derivatives. Spectroscopic properties of these products are similar to the substrate.
8. Data represent the substrate component of this kit.
9. Acetylation by chloramphenicol acetyltransferase (CAT) yields a 3-acetyl-1-deoxychloramphenicol derivative with similar spectroscopic properties to the substrate.
10. ATP-dependent oxidation of luciferin by luciferase results in bioluminescence (Em = 560 nm) at neutral and alkaline pH. Bioluminescence is red-shifted (Em = 617 nm) under acidic conditions (J Am Chem Soc 88, 2015 (1966)).
11. All photoactivatable probes are sensitive to light. They should be protected from illumination except when photolysis is intended.
12. L-7085 is converted to bioluminescent luciferin (L-2911) on ultraviolet photolysis.
13. This product is specified to equal or exceed 98% analytical purity by HPLC.

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